

Certificate of Mailing

Date of Deposit August 24, 2001

Label Number: EL714747741US

I hereby certify under 37 C.F.R. § 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above and is addressed to BOX PATENT APPLICATION, Assistant Commissioner for Patents, Washington, D.C. 20231.

Guy Beardsley
Printed name of person mailing correspondence

Guy Beardsley
Signature of person mailing correspondence

APPLICATION
FOR
UNITED STATES LETTERS PATENT

APPLICANT : BRIAN SEED, BABAK BANAPOUR, CHARLES ROMEO,
AND WALDEMAR KOLANUS

TITLE : TARGETED CYTOLYSIS OF HIV-INFECTED CELLS BY
CHIMERIC CD4 RECEPTOR-BEARING CELLS

009999-08401 2E56E66D

TARGETED CYTOLYSIS OF HIV-INFECTED CELLS BY CHIMERIC
CD4 RECEPTOR-BEARING CELLS

Field of the Invention

10 This application is a continuation-in-part of Seed
et al., U.S.S.N. 08/195,395, filed February 14, 1994, which
is a continuation-in-part of Seed et al., U.S.S.N.
07/847,566, filed March 6, 1992, which is a continuation-in-
part of Seed et al., U.S.S.N. 07/665,961, filed March 7,
1991, now abandoned.

The invention concerns functional chimeras between
CD4 fragments and immune cell receptors which are capable of
directing immune cells to lyse HIV-infected cells, but which
do not render the immune cells susceptible to HIV infection.
The invention therefore provides a novel and effective HIV
therapeutic.

Background of the Invention

25 T cell recognition of antigen through the T cell
receptor is the basis of a range of immunological phenomena.
The T cells direct what is called cell-mediated immunity.
This involves the destruction by cells of the immune system
of foreign tissues or infected cells. A variety of T cells
exist, including "helper" and "suppressor" cells, which
modulate the immune response, and cytotoxic (or "killer")
cells, which can kill abnormal cells directly.

30 A T cell that recognizes and binds a unique antigen
displayed on the surface of another cell becomes activated;
it can then multiply, and if it is a cytotoxic cell, it can
kill the bound cell.

HIV and Immunopathogenesis

In 1984 HIV was shown to be the etiologic agent of AIDS. Since that time the definition of AIDS has been revised a number of times with regard to what criteria should be included in the diagnosis. However, despite the fluctuation in diagnostic parameters, the simple common denominator of AIDS is the infection with HIV and subsequent development of persistent constitutional symptoms and AIDS-defining diseases such as a secondary infections, neoplasms, and neurologic disease. Harrison's Principles of Internal Medicine, 12th ed., McGraw Hill (1991).

HIV is a human retrovirus of the lentivirus group. The four recognized human retroviruses belong to two distinct groups: the human T lymphotropic (or leukemia) retroviruses, HTLV-1 and HTLV-2, and the human immunodeficiency viruses, HIV-1 and HIV-2. The former are transforming viruses whereas the latter are cytopathic viruses.

HIV-1 has been identified as the most common cause of AIDS throughout the world. Sequence homology between HIV-2 and HIV-1 is about 40% with HIV-2 being more closely related to some members of a group of simian immunodeficiency viruses (SIV). See Curran, J. et al., Science, 329:1357-1359 (1985); Weiss, R. et al., Nature, 324:572-575 (1986).

HIV has the usual retroviral genes (env, gag, and pol) as well as six extra genes involved in the replication and other biologic activities of the virus. As stated previously, the common denominator of AIDS is a profound immunosuppression, predominantly of cell-mediated immunity. This immune suppression leads to a variety of opportunistic diseases, particularly certain infections and neoplasms.

The main cause of the immune defect in AIDS has been identified as a quantitative and qualitative deficiency in

the subset of thymus-derived (T) lymphocytes, the T4 population. This subset of cells is defined phenotypically by the presence of the CD4 surface molecule, which has been demonstrated to be the cellular receptor for HIV. Dalglish et al., Nature 312:763 (1984). Although the T4 cell is the major cell type infected with HIV, essentially any human cell that expresses the CD4 molecule on its surface is capable of binding to and being infected with HIV.

Traditionally, CD4⁺ T cells have been assigned the role of helper/inducer, indicating their function in providing an activating signal to B cells, or inducing T lymphocytes bearing the reciprocal CD8 marker to become cytotoxic/suppressor cells. Reinherz and Schlossman, Cell 19:821-827 (1980); Goldstein et al., Immunol. Rev. 68:5-42 (1982).

HIV binds specifically and with high affinity, via a stretch of amino acids in the viral envelope (gp120), to a portion of the V1 region of the CD4 molecule located near its N-terminus. Following binding, the virus fuses with the target cell membrane and is internalized. Once internalized it uses the enzyme reverse transcriptase to transcribe its genomic RNA to DNA, which is integrated into the cellular DNA where it exists for the life of the cell as a "provirus."

The provirus may remain latent or be activated to transcribe mRNA and genomic RNA, leading to protein synthesis, assembly, new virion formation, and budding of virus from the cell surface. Although the precise mechanism by which the virus induces cell death has not been established, it is believed that the major mechanism is massive viral budding from the cell surface, leading to disruption of the plasma membrane and resulting osmotic disequilibrium.

During the course of the infection, the host organism develops antibodies against viral proteins, including the major envelope glycoproteins gp120 and gp41. Despite this humoral immunity, the disease progresses, resulting in a lethal immunosuppression characterized by multiple opportunistic infections, parasitemia, dementia, and death. The failure of the host anti-viral antibodies to arrest the progression of the disease represents one of the most vexing and alarming aspects of the infection, and augurs poorly for vaccination efforts based upon conventional approaches.

Two factors may play a role in the efficacy of the humoral response to immunodeficiency viruses. First, like other RNA viruses (and like retroviruses in particular), the immunodeficiency viruses show a high mutation rate in response to host immune surveillance. Second, the envelope glycoproteins themselves are heavily glycosylated molecules presenting few epitopes suitable for high affinity antibody binding. The poorly antigenic target which the viral envelope presents allows the host little opportunity for restricting viral infection by specific antibody production.

Cells infected by the HIV virus express the gp120 glycoprotein on their surface. Gp120 mediates fusion events among CD4⁺ cells via a reaction similar to that by which the virus enters the uninfected cells, leading to the formation of short-lived multinucleated giant cells. Syncytium formation is dependent on a direct interaction of the gp120 envelope glycoprotein with the CD4 protein. Dalglish et al., supra; Klatzman, D. et al., Nature 312:763 (1984); McDougal, J.S. et al., Science 231:382 (1986); Sodroski, J. et al., Nature 322:470 (1986); Lifson, J.D. et al., Nature 323:725 (1986); Sodroski, J. et al., Nature 321:412 (1986).

Evidence that the CD4-gp120 binding is responsible for viral infection of cells bearing the CD4 antigen includes the finding that a specific complex is formed between gp120 and CD4. McDougal et al., supra. Other
5 investigators have shown that the cell lines, which were noninfective for HIV, were converted to infectable cell lines following transfection and expression of the human CD4 cDNA gene. Maddon et al., Cell 46:333-348 (1986).

Therapeutic programs based on soluble CD4 as a
10 passive agent to interfere with viral adsorption and syncytium-mediated cellular transmission have been proposed and successfully demonstrated in vitro by a number of groups (Deen et al., Nature 331:82-84 (1988); Fisher et al., Nature 331:76-78 (1988); Hussey et al., Nature 331:78-81 (1988);
15 Smith et al., Science 238:1704-1707 (1987); Trauneker et al., Nature 331:84-86 (1988)); and CD4 immunoglobulin fusion proteins with extended halflives and modest biological activity have subsequently been developed (Capon et al., Nature 337:525-531 (1989); Trauneker et al. Nature 339, 68-
20 70 (1989); Byrn et al., Nature 344:667-670 (1990); Zettlmeissl et al., DNA Cell Biol. 9:347-353 (1990)). Although CD4 immunotoxin conjugates or fusion proteins show potent cytotoxicity for infected cells in vitro (Chaudhary et al., Nature 335:369-372 (1988); Till et al., Science
25 242:1166-1168 (1988)), the latency of the immunodeficiency syndrome makes it unlikely that any single-treatment therapy will be effective in eliminating viral burden, and the antigenicity of foreign fusion proteins is likely to limit their acceptability in treatments requiring repetitive
30 dosing. Trials with monkeys affected with SIV have shown that soluble CD4, if administered to animals without marked CD4 cytopenia, can reduce SIV titer and improve in vitro measures of myeloid potential (Watanabe et al., Nature

FOI 30 254560

337:267-270 (1989)). However a prompt viral reemergence was observed after treatment was discontinued, suggesting that lifelong administration might be necessary to prevent progressive immune system debilitation.

5 T Cell and Fc Receptors

Cell surface expression of the most abundant form of the T cell antigen receptor (TCR) requires the coexpression of at least 6 distinct polypeptide chains (Weiss et al., J. Exp. Med. 160:1284-1299 (1984); Orloffhashi et al., Nature 316:606-609 (1985); Berkhout et al., J. Biol. Chem. 263:8528-8536 (1988); Sussman et al., Cell 52:85-95 (1988)), the α/β antigen binding chains, the three polypeptides of the CD3 complex, and ζ . If any of the chains are absent, stable expression of the remaining members of the complex does not ensue. ζ is the limiting polypeptide for surface expression of the complete complex (Sussman et al., Cell 52:85-95 (1988)) and is thought to mediate at least a fraction of the cellular activation programs triggered by receptor recognition of ligand (Weissman et al., EMBO J. 8:3651-3656 (1989); Frank et al., Science 249:174-177 (1990)). A 32kDa type I integral membrane homodimer, ζ (zeta) has a 9 residue extracellular domain with no sites for N-linked glycan addition, and a 112 residue (mouse) or 113 residue (human) intracellular domain (Weissman et al., Science 238:1018-1020 (1988); Weissman et al., Proc. Natl. Acad. Sci. USA 85:9709-9713 (1988)). An isoform of ζ called η (eta) (Baniyash et al., J. Biol. Chem. 263:9874-9878 (1988); Orloff et al., J. Biol. Chem. 264:14812-14817 (1989)), which arises from an alternate mRNA splicing pathway (Jin et al., Proc. Natl. Acad. Sci. USA 87:3319-3233 (1990)), is present in reduced amounts in cells expressing

the antigen receptor. ζ - η heterodimers are thought to mediate the formation of inositol phosphates, as well as the receptor-initiated programmed cell death called apoptosis (Merćep et al., Science 242:571-574 (1988); Merćep et al., Science 246:1162-1165 (1989)).

Like ζ and η , the Fc receptor-associated γ chain is expressed in cell surface complexes with additional polypeptides, some of which mediate ligand recognition, and others of which have undefined function. γ (gamma) bears a homodimeric structure and overall organization very similar to that of ζ and is a component of both the mast cell/basophil high affinity IgE receptor, Fc ϵ RI, which consists of at least three distinct polypeptide chains (Blank et al., Nature 337:187-189 (1989); Ra et al., Nature 241:752-754 (1989)), and one of the low affinity receptors for IgG, represented in mice by Fc γ RII α (Ra et al., J. Biol. Chem. 264:15323-15327 (1989)), and in humans by the CD16 subtype expression by macrophages and natural killer cells, CD16_{TM} (CD16 transmembrane) (Lanier et al., Nature 342:803-805 (1989); Anderson et al., Proc. Natl. Acad. Sci. USA 87:2274-2278 (1990)) and with a polypeptide of unidentified function (Anderson et al., Proc. Natl. Acad. Sci. USA 87:2274-2278 (1990)). Recently it has been reported that γ is expressed by a mouse T cell line, CTL, in which it forms homodimers as well as γ - ζ and γ - η heterodimers (Orloff et al., Nature 347:189-191 (1990)).

The Fc receptors mediate phagocytosis of immune complexes, transcytosis, and antibody dependent cellular cytotoxicity (ADCC) (Ravetch and Kinet, Annu. Rev. Immunol. 9:457-492 (1991); Unkeless et al., Annu. Rev. Immunol. 6:251-281 (1988); and Mellman, Curr. Opin. Immunol. 1:16-25 (1988)). Recently it has been shown that one of the murine low affinity Fc receptor isoforms, FcR γ IIIB1, mediates

internalization of Ig-coated targets into clathrin coated pits, and that another low affinity receptor, FcγIIIA mediates ADCC through its association with one or more members of a small family of 'trigger molecules' (Miettinen et al., Cell 58:317-327 (1989); and Hunziker and Mellman, J. Cell Biol. 109:3291-3302 (1989)). These trigger molecules, T cell receptor (TCR) ζ chain, TCR η chain, and Fc receptor γ chain interact with ligand recognition domains of different immune system receptors and can autonomously initiate cellular effector programs, including cytolysis, following aggregation (Sanzel et al., Cell 43:223-231 (1985); Weissman et al., Science 239:1018-1020 (1988); Jin et al., Proc. Natl. Acad. Sci. USA 87:3319-3323 (1990); Blank et al., Nature 337:187-189 (1989); Lanier et al., Nature 342:803-805 (1989); Kurosaki and Ravetch, Nature 342:805-807 (1989); Hibbs et al., Science 246:1608-1611 (1989); Anderson et al., Proc. Natl. Acad. Sci. USA 87:2274-2278 (1990); and Irving and Weiss, Cell 64: 891-901 (1991)).

In drawing parallels between the murine and human low affinity Fc receptor families, however, it has become clear that the human FcγIIA and C isoforms have no murine counterpart. In part because of this, their function has yet to be defined.

Because humoral agents based on CD4 alone may have limited utility in vivo, previous work explored the possibility of augmenting cellular immunity to HIV. Preparations of protein chimeras in which the extracellular domain of CD4 is fused to the transmembrane and/or intracellular domains of T cell receptor, IgG Fc receptor, or B cell receptor signal transducing elements have been identified (U.S.S.N. 07/847,566 and 07/665,961, hereby incorporated by reference). Cytolytic T cells expressing chimeras which include an extracellular CD4 domain show

potent MHC-independent destruction of cellular targets expressing HIV envelope proteins. An extremely important and novel component of this approach has been the identification of single T cell receptor, Fc receptor, and B cell receptor chains whose aggregation suffices to initiate the cellular response. One particularly useful application of this approach has been the invention of chimeras between CD4 and ζ , η , or γ that direct cytolytic T lymphocytes to recognize and kill cells expressing HIV gp120 (U.S.S.N. 07/847,566 and 07/665,961, hereby incorporated by reference).

Summary of the Invention

In general, the invention features a method of directing a cellular immune response against an HIV-infected cell in a mammal. The method involves administering to the mammal an effective amount of therapeutic cells, the therapeutic cells expressing a membrane-bound, proteinaceous chimeric receptor comprising (a) an extracellular portion which includes a fragment of CD4 which is capable of specifically recognizing and binding the HIV-infected cell but which does not mediate HIV infection and (b) an intracellular portion which is capable of signalling the therapeutic cell to destroy the receptor-bound HIV-infected cell.

In a second aspect, the invention features a cell which expresses a proteinaceous membrane-bound chimeric receptor which comprises (a) an extracellular portion which includes a fragment of CD4 which is capable of specifically recognizing and binding the HIV-infected cell but which does not mediate HIV infection and (b) an intracellular portion which is capable of signalling the therapeutic cell to destroy the receptor-bound HIV-infected cell.

In preferred embodiments of both aspects, the CD4 fragment is amino acids 1-394 of CD4 or is amino acids 1-200 of CD4; the CD4 fragment is separated from the intracellular portion by the CD7 transmembrane domain shown in Fig. 26 or by the hinge, CH2, and CH3 domains of the human IgG1 molecule shown in Fig. 25; the intracellular portion is the signal-transducing portion of a T cell receptor protein (for example, ζ), a B cell receptor protein, or an Fc receptor protein; and the therapeutic cells are selected from the group consisting of: (a) T lymphocytes; (b) cytotoxic T lymphocytes; (c) natural killer cells; (d) neutrophils; (e) granulocytes; (f) macrophages; (g) mast cells; (h) HeLa cells; and (i) embryonic stem cells (ES).

In other aspects, the invention features DNA encoding a chimeric receptor of the invention; and a vector including that chimeric receptor DNA.

Although the specific embodiment of the present invention is a chimera between CD4 and zeta, any receptor chain having a similar function to these molecules, e.g., in granulocytes or B lymphocytes, could be used for the purposes disclosed here. The distinguishing features of a desirable immune cell trigger molecule comprises the ability to be expressed autonomously (i.e., as a single chain), the ability to be fused to an extracellular CD4 domain such that the resultant chimera is present on the surface of a therapeutic cell, and the ability to initiate cellular effector programs upon aggregation secondary to encounter with a target ligand.

At present the most convenient method for delivery of the chimeras to immune system cells is through some form of genetic therapy. However reconstituting immune system cells with chimeric receptors by mixture of the cells with suitably solubilized purified chimeric protein would also

result in the formation of an engineered cell population capable of responding to HIV-infected targets. Similar approaches have been used, for example, to introduce the CD4 molecule into erythrocytes for therapeutic purposes. In this case the engineered cell population would not be capable of self renewal.

The present invention relates to functional and simplified chimeras between CD4 fragments and T cell receptor, B cell receptor, and Fc receptor subunits which are capable of directing immune cells to recognize and lyse HIV-infected cells. The method for directing the cellular response in a mammal comprises administering an effective amount of therapeutic cells (for example, cytotoxic T lymphocytes) to the mammal, the cells being capable of recognizing and destroying the HIV-infected cell.

The invention also includes the chimeric receptor proteins which direct the cytotoxic T lymphocytes to recognize and lyse HIV-infected cells, the host cells transformed with a vector comprising the chimeric receptors, and antibodies directed against the chimeric receptors.

These and other non-limiting embodiments of the present invention will be apparent to those of skill from the following detailed description of the invention.

In the following detailed description, reference will be made to various methodologies known to those of skill in the art of molecular biology and immunology. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference in their entireties as though set forth in full.

Standard reference works setting forth the general principles of recombinant DNA technology include Watson et al., Molecular Biology of the Gene, Volumes I and II, the

Benjamin/Cummings Publishing Company, Inc., publisher, Menlo Park, CA (1987); Darnell et al., Molecular Cell Biology, Scientific American Books, Inc., Publisher, New York, N.Y. (1986); Lewin, Genes II, John Wiley & Sons, publishers, New York, N.Y. (1985); Old et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2d edition, University of California Press, publisher, Berkeley, CA (1981); Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory, publisher, Cold Spring Harbor, NY (1989); and Ausubel et al., Current Protocols in Molecular Biology, Wiley Press, New York, NY (1989).

DEFINITIONS

By "cloning" is meant the use of in vitro recombination techniques to insert a particular gene or other DNA sequence into a vector molecule.

By "cDNA" is meant complementary or copy DNA produced from an RNA template by the action of RNA-dependent DNA polymerase (reverse transcriptase). Thus a "cDNA clone" means a duplex DNA sequence complementary to an RNA molecule of interest, carried in a cloning vector.

By "cDNA library" is meant a collection of recombinant DNA molecules containing cDNA inserts which comprise DNA copies of mRNA being expressed by the cell at the time the cDNA library was made. Such a cDNA library may be prepared by methods known to those of skill, and described, for example, in Ausubel et al., supra and Maniatis et al., supra. Generally, RNA is first isolated from the cells of an organism from whose genome it is desired to clone a particular gene. Preferred for the purpose of the present invention are mammalian, and particularly human, lymphocytic cell lines. A presently

preferred vector for this purpose is the vaccinia virus WR strain.

By "vector" is meant a DNA molecule, derived, e.g., from a plasmid, bacteriophage, or mammalian or insect virus, into which fragments of DNA may be inserted or cloned. A vector will contain one or more unique restriction sites and may be capable of autonomous replication in a defined host or vehicle organism such that the cloned sequence is reproducible. Thus, by "DNA expression vector" is meant any autonomous element capable of directing the synthesis of a recombinant peptide. Such DNA expression vectors include bacterial plasmids and phages and mammalian and insect plasmids and viruses.

By "substantially pure" is meant a compound, e.g., a protein, a polypeptide, or an antibody, that is substantially free of the components that naturally accompany it. Generally, a compound is substantially pure when at least 60%, more preferably at least 75%, and most preferably at least 90% of the total material in a sample is the compound of interest. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. In the context of a nucleic acid, "substantially pure" means a nucleic acid sequence, segment, or fragment that is not immediately contiguous with (i.e., covalently linked to) both of the coding sequences with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally occurring genome of the organism from which the DNA of the invention is derived.

A "fragment" of a molecule, such as any of the cDNA sequences of the present invention, is meant to refer to any contiguous nucleotide subset of the molecule. An "analog" of a molecule is meant to refer to a non-natural molecule

substantially similar to either the entire molecule or a fragment thereof. A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same. Substantially similar amino acid molecules will possess a similar biological activity. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, Penn. (1980).

A "functional derivative" of a receptor chimera gene of the present invention is meant to include "fragments" or "analogues" of the gene, which are "substantially similar" in nucleotide sequence, and which encode a molecule possessing similar activity to, for example, a T cell, B cell, or Fc receptor chimera. Most preferably, the derivative possesses 90%, more preferably, 70%, and preferably 40% of the wild-type receptor chimera's activity.

The activity of a functional chimeric receptor derivative includes specific binding (with its extracellular CD4 portion) to an HIV-infected cell and resultant destruction of that cell; in addition, the chimeric receptor does not render the receptor-bearing cell susceptible to HIV infection. Chimeric receptor activity may be tested using, e.g., any of the assays described herein.

A DNA sequence encoding the CD4 receptor chimera of the present invention, or its functional derivatives, may be

recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed by Maniatis, T., et al., supra, and are well known in the art.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

If desired, the non-coding region 3' to the gene sequence coding for the protein may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the DNA sequence coding for the protein, the transcriptional termination signals may

be provided. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted.

5 Two DNA sequences (such as a promoter region sequence and a CD4-receptor chimera encoding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with
10 the ability of the promoter region sequence to direct the transcription of the receptor chimera gene sequence, or (3) interfere with the ability of the receptor chimera gene sequence to be transcribed by the promoter region sequence. A promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. Thus, to express the protein, transcriptional and translational signals recognized by an appropriate host are necessary.

FOI 280 25660
10420
The present invention encompasses the expression of a CD4-receptor chimera protein (or a functional derivative thereof) in either prokaryotic or eukaryotic cells, although eukaryotic (and, particularly, human lymphocyte) expression is preferred.

25 Antibodies according to the present invention may be prepared by any of a variety of methods. For example, cells expressing the CD4-receptor chimera protein, or a functional derivative thereof, can be administered to an animal in order to induce the production of sera containing polyclonal antibodies that are capable of binding the chimera.

30 In a preferred method, antibodies according to the present invention are monoclonal antibodies. Such monoclonal antibodies can be prepared using hybridoma technology (Kohler et al., Nature 256:495 (1975); Kohler et

Standard reference works setting forth general principles of immunology include Roitt, Essential Immunology, 6th ed., Blackwell Scientific Publications, Publisher, Oxford (1958); Kimball, Introduction to Immunology, 2d ed., Macmillan Publishing Co., Publisher, New

York (1986); Roitt et al., Immunology, Gower Medical Publishing Ltd., Publisher, London, (1985); Campbell, "Monoclonal Antibody Technology," in Burdon et al., eds., Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier, Publisher, Amsterdam (1984); Klein, Immunology: The Science of Self-Nonself Discrimination, John Wiley & Sons, Publisher, New York (1982); and Kennett et al., eds., Monoclonal Antibodies, Hybridoma: A New Dimension In Biological Analyses, Plenum Press, Publisher, New York (1980).

By "detecting" it is intended to include determining the presence or absence of a substance or quantifying the amount of a substance. The term thus refers to the use of the materials, compositions, and methods of the present invention for qualitative and quantitative determinations.

The antibodies and substantially purified antigen of the present invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement therewith one or more container means such as vials, tubes and the like, each of said container means comprising the separate elements of the assay to be used.

The types of assays which can be incorporated in kit form are many, and include, for example, competitive and non-competitive assays. Typical examples of assays which can utilize the antibodies of the invention are radioimmunoassays (RIA), enzyme immunoassays (EIA), enzyme-linked immunoadsorbent assays (ELISA), and immunometric, or sandwich, immunoassays.

By the term "immunometric assay" or "sandwich immunoassay," it is meant to include simultaneous sandwich, forward sandwich, and reverse sandwich immunoassays. These terms are well understood by those skilled in the art.

Those of skill will also appreciate that antibodies according to the present invention will be useful in other variations and forms of assays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

By "specifically recognizes and binds" is meant that the antibody recognizes and binds the chimeric receptor polypeptide but does not substantially recognize and bind other unrelated molecules in a sample, e.g., a biological sample.

By "therapeutic cell" is meant a cell which has been transformed by a CD4-receptor chimera of the invention so that it is capable of recognizing and destroying an HIV-infected cell; preferably such therapeutic cells are cells of the hematopoietic system.

By "extracellular" is meant having at least a portion of the molecule exposed at the cell surface. By "intracellular" is meant having at least a portion of the molecule exposed to the therapeutic cell's cytoplasm. By "transmembrane" is meant having at least a portion of the molecule spanning the plasma membrane. An "extracellular portion", an "intracellular portion" and a "transmembrane portion", as used herein, may include flanking amino acid sequences which extend into adjoining cellular compartments.

By "oligomerize" is meant to complex with other proteins to form dimers, trimers, tetramers, or other higher order oligomers. Such oligomers may be homo-oligomers or hetero-oligomers. An "oligomerizing portion" is that region of a molecule which directs complex (i.e., oligomer) formation.

By "cytolytic" is meant to be capable of destroying a cell (e.g., an HIV-infected cell) or to be capable of destroying an infective agent (e.g., an HIV virus).

By "immunodeficiency virus" is meant a retrovirus that, in wild-type form, is capable of infecting T4 cells of a primate host and possesses a viral morphogenesis and morphology characteristic of the lentivirus subfamily. The term includes, without limitation, all variants of HIV and SIV, including HIV-1, HIV-2, SIVmac, SIVagm, SIVmnd, SIVsmm, SIVman, SIVmand, and SIVcpz.

By "MHC-independent" is meant that the cellular cytolytic response does not require the presence of an MHC class II antigen on the surface of the targeted cell.

By a "functional cytolytic signal-transducing derivative" is meant a functional derivative (as defined above) which is capable of directing at least 40%, more preferably 70%, or most preferably at least 90% of the biological activity of the wild type molecule. As used herein, a "functional cytolytic signal-transducing derivative" may act by directly signaling the therapeutic cell to destroy a receptor-bound agent or cell (e.g., in the case of an intracellular chimeric receptor portion) or may act indirectly by promoting oligomerization with cytolytic signal transducing proteins of the therapeutic cell (e.g., in the case of a transmembrane domain). Such derivatives may be tested for efficacy, e.g., using the in vitro assays described herein.

By a "functional HIV envelope-binding derivative" is meant a functional derivative (as defined above) which is capable of binding any HIV envelope protein. Functional derivatives may be identified using, e.g., the in vitro assays described herein.

THERAPEUTIC ADMINISTRATION

The transformed cells of the present invention are used for immunodeficiency virus therapy. Current methods of

administering such transformed cells involve adoptive immunotherapy or cell-transfer therapy. These methods allow the return of the transformed immune-system cells to the bloodstream. Rosenberg, Scientific American 62 (May 1990);
5 Rosenberg et al., The New England Journal of Medicine 323(9):570 (1990).

The pharmaceutical compositions of the invention may be administered to any animal which may experience the beneficial effects of the compounds of the invention.

10 Foremost among such animals are humans, although the invention is not intended to be so limited.

Detailed Description

The drawings will first be described.

Brief Description of the Drawings

Sub
A2
FIG. 1A presents the amino acid sequence about the site of fusion between CD4 (residues 1-369) and different receptor chains. The underlined sequence shows the position of the amino acids encoded within the BamHI site used for fusion construction. The beginning of the transmembrane domain is marked with a vertical bar. The η sequence is identical to the ζ sequence at the amino terminus, but diverges at the carboxyl terminus (Jin et al., Proc. Natl. Acad. Sci. USA 87:3319-3323 (1990)). FIG. 1B presents flow cytometric analysis of surface expression of CD4, CD4: ζ ,
20 CD4: γ and CD4: η in CV1 cells. Cells were infected with virus expressing CD4 chimeras or CD16_{PI}, incubated for 9 hours at 37°C, and stained with phycoerythrin-conjugated anti-CD4 MAb Leu3A.

FIG. 2 shows surface expression of CD16_{TM} following
30 coinfection of CD16_{TM} alone (dense dots), or coinfecting with virus expressing CD4: γ (dashes) or CD4: ζ (solid line).

Sparse dots, cells infected with CD4:ζ alone, stained with 3G8 (Fleit et al., Proc. Natl. Acad. Sci. USA 79:3275-3279 (1982)) (anti-CD16 MAb).

FIG. 3 shows surface expression of CD16_{TM} following coinfection by viruses expressing CD16_{TM} and the following ζ chimeras: CD4:ζ (thick line), CD4:ζ C11G (solid line); CD4:ζ (dashed line); CD4:ζ C11G/D15G (dense dots); no coinfection (CD16_{TM} alone, sparse dots). Cells were incubated with anti-CD16 MAb 3G8 and phycoerythrin-conjugated Fab'₂ goat antibodies to mouse IgG. The level of expression of the ζ chimeras was essentially identical for the different mutants analyzed, and coinfection of cells with viruses expressing CD16_{TM} and ζ chimeras did not appreciably alter surface expression of the chimeras.

FIG. 4A-D shows increased intracellular free calcium ion follows crosslinking of mutant ζ chimeras in a T cell line. Jurkat E6 cells (Weiss et al., J. Immunol. 133:123-128 (1984)) were infected with recombinant vaccinia viruses and analyzed by flow cytometry. The results shown are for the gated CD4⁺ population, so that only cells expressing the relevant chimeric protein are analyzed. The mean ratio of violet to blue Indo-1 fluorescence reflects the intracellular free calcium concentration in the population as a whole and the percentage of responding cells reflects the fraction of cells which exceed a predetermined threshold ratio (set so that 10% of untreated cells are positive).

FIG. 4A and FIG. 4B show Jurkat cells expressing CD4:ζ (solid line) or CD16:ζ (dashed line) which were exposed to anti-CD4 MAb Leu3a (phycoerythrin conjugate), followed by crosslinking with goat antibody to mouse IgG. The dotted line shows the response of uninfected cells to anti-CD3 MAb OKT3. FIGS. 4C and 4D show Jurkat cells expressing CD4:ζD15G (solid line); CD4:ζC11G/D15G (dashes); or

CD4:ζC11G (dots) which were treated and analyzed as in FIGS. 4A and 4B.

FIG. 5A-C shows that CD4:ζ, CD4:η, and CD4:γ receptors allow cytolytic T lymphocytes (CTL) to kill targets expressing HIV-1 gp120/41. FIG. 5A: solid circles, CTL expressing CD4:ζ incubated with HeLa cells expressing gp120/41; open circles, CTL expressing CD4:ζ incubated with uninfected HeLa cells; solid squares, uninfected CTL incubated with HeLa cells expressing gp120/41; open squares, uninfected CTL incubated with uninfected HeLa cells. FIG. 5B: solid circles, CTL expressing CD4:η incubated with HeLa cells expressing gp120/41; open circles, CTL expressing CD4:γ incubated with HeLa cells expressing gp120/41; open squares, CTL expressing the C11G/D15G double mutant CD4:ζ chimera incubated with HeLa cells expressing gp120/41. FIG. 5C: Flow cytometric analysis of CD4 expression by the CTL used in Fig. 5B. To correct the target to effector ratios the percent of cells expressing CD4 chimera was determined by subtracting the scaled negative (uninfected) population by histogram superposition; for comparative purposes in this figure the uninfected cells were assigned an arbitrary threshold which gives roughly the same fraction positive for the other cell populations as would histogram subtraction.

FIG. 6A-B shows specificity of CD4-directed cytotoxicity. FIG. 6A: solid circles, CTL expressing CD4:ζ incubated with HeLa cells expressing CD16_{p1}; open circles, CTL expressing CD4 incubated with HeLa cells expressing gp120; solid squares, CTL expressing CD16:ζ incubated with HeLa cells expressing gp120/41; open squares, CTL expressing CD16_{p1} incubated with HeLa cells expressing gp120/41. FIG. 6B: solid circles, CTL expressing CD4:ζ incubated with Raji (MHC class II⁺) cells; open circles, uninfected CTL cells incubated with RJ2.2.5 (MHC class II⁻ Raji mutant)

cells; solid squares, uninfected CTL incubated with Raji (MHC class II⁺) cells; open squares, CTL expressing CD4:ζ incubated with RJ2.2.5 (MHC class II⁻) cells. The ordinate scale is expanded.

5 **FIG. 7A-B** shows characterization of the CD16:ζ chimeric receptor. **FIG. 7A** is a schematic diagram of the CD16:ζ fusion protein. The extracellular portion of the phosphatidylinositol-linked form of monomeric CD16 was joined to dimeric ζ just external to the transmembrane domain. The protein sequence at the fusion junction is shown at the bottom. **FIG. 7B** shows a flow cytometric analysis of calcium mobilization following crosslinking of the CD16:ζ chimera in either a TCR positive or TCR negative cell line. The mean ratio of violet to blue fluorescence (a measure of relative calcium ion concentration) among cell populations treated with antibodies at time 0 is shown. Solid squares, the response of Jurkat cells to anti-CD3 MAb OKT3; solid triangles, the response of CD16:ζ to anti-CD16 MAb 3G8 crosslinking in the REX33A TCR⁻ mutant; open squares, the response to CD16:ζ crosslinking in the Jurkat TCR⁻ mutant line JRT3.T3.5; open triangles, the response to CD16:ζ crosslinking in Jurkat cells; crosses, the response to nonchimeric CD16 in Jurkat cells; and dots, the response to nonchimeric CD16 in the REX33A TCR⁻ cell line.

25 **FIG. 8A-B** shows deletion analysis of cytolytic potential. **FIG. 8A** shows the locations of the ζ deletion endpoints. Here as elsewhere mutations in ζ are represented by the original residue-location-mutant residue convention, so that D66*, for example, denotes replacement of Asp-66 by a termination codon. **FIG. 8B** shows cytotoxicity assay results of undeleted CD16:ζ and salient ζ deletions. Hybridoma cells expressing surface antibody to CD16 were loaded with ⁵¹Cr and incubated with increasing numbers of human

cytolytic lymphocytes (CTL) infected with vaccinia recombinants expressing CD16:ζ chimeras. The percent of ⁵¹Cr released is plotted as a function of the effector (CTL) to target (hybridoma) cell ratio (e/t). Solid circles, 5 cytotoxicity mediated by cells expressing CD16:ζ (mfi 18.7); solid squares, cytotoxicity mediated by cells expressing CD16:ζ Asp66* (mfi 940.2); open squares, cytotoxicity mediated by cells expressing CD16:ζGlu60* (mfi 16.0); open circles, 10 cytotoxicity mediated by cells expressing CD16:ζTyr51* (mfi 17.4); solid triangles, cytotoxicity mediated by cells expressing CD16:ζPhe34* (mfi 17.8); and open triangles, cytotoxicity mediated by cells expressing nonchimeric CD16 (mfi 591). Although in this experiment the expression of CD16:ζAsp66* was not matched to that of the other fusion proteins, cytotoxicity by cells expressing CD16:ζ at equivalent levels in the same experiment gave results essentially identical to those shown by cells expressing CD16:ζAsp66.

FIG. 9A-D shows that elimination of the potential for transmembrane interactions reveals a short ζ segment capable of mediating cytotoxicity. FIG. 9A is a schematic diagram of the monomeric bipartite and tripartite chimeras. At the top is the CD16:ζ construct truncated at residue 65 and lacking transmembrane Cys and Asp residues. Below are the CD16:CD5:ζ and CD16:CD7:ζ constructs and related 25 controls. The peptide sequences of the intracellular domains are shown below. FIG. 9B shows the cytotoxic activity of monomeric chimera deletion mutants. The cytotoxic activity of cells expressing CD16:ζ (solid circles; mfi 495) was compared to that of cells expressing CD16:ζAsp66* (solid squares; mfi 527) or the mutants 30 CD16:ζCys11Gly/Asp15Gly/Asp66*, (open squares; mfi 338) and CD16:ζCys11Gly/Asp15Gly/Glu60* (filled triangles; mfi 259). FIG. 9C shows the cytotoxic activity mediated by tripartite

009337 250560
FOI 280 08401

Sub
Q4

fusion proteins. Solid triangles, CD16:ζAsp66*; open squares, CD16:5:ζ(48-65); solid squares CD16:7:ζ(48-65); open triangles, CD16:7:ζ(48-59); open circles, CD16:5; solid circles, CD16:7. FIG. 9D shows calcium mobilization by mutant and tripartite chimeras in the TCR negative Jurkat JRT3.T3.5 mutant cell line. Open circles, response of cells expressing dimeric CD16:ζAsp66*; solid squares, response of cells expressing CD16:ζCys11Gly/Asp15Gly/Asp66*; open squares, response of cells expressing CD16:ζCys11Gly/Asp15Gly/Glu60*; solid triangles, response of cells expressing CD16:7:ζ(48-65); and open triangles, response of cells expressing CD16:ζ(48-59).

FIG. 10A-F shows the contribution of individual amino acids to the activity of the 18 residue cytolytic signal-transducing motif. FIGS. 10A and 10B show cytolytic activity and FIG. 10C shows calcium ion mobilization mediated by chimeras bearing point mutations near the carboxyl terminal tyrosine (Y62). FIGS. 10A and 10B represent data collected on cells expressing low and high amounts, respectively, of the CD16:ζ fusion proteins. Identical symbols are used for the calcium mobilization and cytotoxicity assays, and are shown in one letter code at right. Solid circles, cells expressing CD16:ζ (mfi in A, 21; B, 376); solid squares, cells expressing CD16:7:ζ(48-65) (mfi A, 31; B, 82); open squares, CD16:7:ζ(48-65)Glu60Gln (mfi A, 33; B, 92), crosses, CD16:7:ζ(48-65)Asp63Asn (mfi A, 30; B, 74); solid triangles, CD16:7:ζ(48-65)Tyr62Phe (mfi A, 24; B, 88); open circles, CD16:7:ζ(48-65)Glu61Gln (mfi A, 20; B, 62); and open triangles, CD16:7:ζ(48-65)Tyr62Ser (mfi B, 64). FIGS. 10D and 10E show cytolytic activity and FIG. 10F shows calcium ion mobilization by chimeras bearing point mutations near the amino terminal tyrosine (Y51). Identical symbols are used for the calcium mobilization and cytotoxicity

assays and are shown at right. Solid circles, cells expressing CD16:ζ (mfi in D, 21.2; in E, 672); solid squares, cells expressing CD16:7:ζ(48-65) (mfi D, 31.3; E, 179); solid triangles, CD16:7:ζ(48-65)Asn48Ser (mfi D, 22.4; E, 209); open squares, CD16:7:ζ(48-65)Leu50Ser (mfi D, 25.0; E, 142); and open triangles, CD16:7:ζ(48-65)Tyr51Phe (mfi D, 32.3; E, 294).

Sub
0.5

10

FIG. 11A-B shows alignment of internal repeats of ζ and comparison of their ability to support cytolysis. FIG. 11A is a schematic diagram of chimeras formed by dividing the ζ intracellular domain into thirds and appending them to the transmembrane domain of a CD16:7 chimera. The sequences of the intracellular domains are shown below, with shared residues boxed, and related residues denoted by asterisks. FIG. 11B shows the cytolytic potency of the three ζ subdomains. Solid circles, cells expressing CD16:ζ (mfi 476); solid squares, CD16:7:ζ(33-65) (mfi 68); open squares, CD16:7:ζ(71-104) (mfi 114); and solid triangles, CD16:7:ζ(104-138) (mfi 104).

FIG. 12 is a schematic diagram of the CD16:FcRγII chimeras.

FIG. 13A-B shows calcium mobilization following crosslinking of CD4:FcRγII and CD16:FcRγII chimeras. FIG. 13A shows the ratio of violet to blue fluorescence emitted by cells loaded with the calcium sensitive fluorophore Indo-1 shown as a function of time following crosslinking of the CD16 extracellular domain with antibodies. FIG. 13B shows a similar analysis of the increase in ratio of violet to blue fluorescence of cells bearing CD4:FcRγII chimeras, following crosslinking with antibodies.

FIG. 14A-B shows cytolysis assays of CD4:FcRγII and CD16:FcRγII chimeras. FIG. 14A shows the percent of ⁵¹Cr released from anti-CD16 hybridoma (target) cells when the

cells are exposed to increasing numbers of cytotoxic T lymphocytes expressing CD16:FcRyII chimeras (effector cells). FIG. 14B shows a similar analysis of cytotoxicity mediated by CD4:FcRyII chimeras against target cells expressing HIV envelope glycoproteins.

FIG. 15A-E shows identification of residues in the FcRyII A tail which are important for cytolysis. FIG. 15A is a schematic diagram of the deletion constructs. FIGS. 15B and 15C shows calcium mobilization and cytolysis by carboxyl-terminal deletion variants of CD16:FcRyII A. FIGS. 15D and 15E show calcium mobilization and cytolysis by tripartite chimeras bearing progressively less of the amino terminus of the intracellular tail of CD16:FcRyII A.

FIG. 16 (SEQ ID NO: 24) shows the amino acid sequence of the CD3 delta receptor protein; the boxed sequence represents a preferred cytolytic signal transducing portion.

FIG. 17 (SEQ ID NO: 25) shows the amino acid sequence of the T3 gamma receptor protein; the boxed sequence represents a preferred cytolytic signal transducing portion.

FIG. 18 (SEQ ID NO: 26) shows the amino acid sequence of the mb1 receptor protein; the boxed sequence represents a preferred cytolytic signal transducing portion.

FIG. 19 (SEQ ID NO: 27) shows the amino acid sequence of the B29 receptor protein; the boxed sequence represents a preferred cytolytic signal transducing portion.

FIG. 20 shows a schematic diagram of the CD4 chimeras. Molecule "A" is CD4(D1-D4):Ig:CD7; molecule "B" is CD4(D1,D2):Ig:CD7; molecule "C" is CD4(D1-D4):Ig:CD7:ζ; molecule "D" is CD4(D1,D2):Ig:CD7:ζ; and molecule "E" is CD4:ζ. The extracellular domain of the human CD4 molecule corresponding to amino acids 1-394 of the precursor was

joined by a BamHI site to the hinge, CH1, and CH2 domains of human IgG1 as described previously (Zettlmeissl et al., DNA Cell Biol. 9:347 (1990)) except that a cDNA version of the human Ig sequences was used to allow expression in vaccinia virus recombinants. The two-domain version of the CD4 chimeras were created by insertion of a BamHI adaptor at the unique NheI site (corresponding to amino acid 200) in the CD4 precursor cDNA. The membrane attachment sequences consisted of 22 residues from the first exon of human membrane-bound IgG1 followed by CD7 residues 146-203. Amino acids 55 through 163 of ζ served as the trigger motif of the tetrapartite constructs (C and D). In tetrapartite constructs containing the ζ chain, intracellular expression of ζ was documented with a commercially available antibody against the intracellular domain (Coulter).

FIG. 21 shows cytolysis of target cells expressing the HIV-1 envelope glycoprotein mediated by the cytotoxic T-cell clone, WH3, expressing various CD4-derived chimeras as effector molecules. For cytotoxicity assays, the human CD8⁺ CD4⁻ HLA B44 restricted T cell line, WH3, was maintained in IMDM supplemented with 10% human serum as previously described herein. The cells were stimulated with gamma-irradiated (3000rad) B44-bearing mononuclear cells and phytohemagglutinin (PHA) at 1 μ g/ml. After one day of stimulation, the PHA was diluted to 0.5 μ g/ml by addition of fresh medium; after 3 days the medium was changed completely. Cells were grown for at least 10 days before use in cytotoxicity assays. Cells were infected with the appropriate recombinant vaccinia viruses as described herein for vPE16. Infections were allowed to proceed for an additional 3-4 hours in complete medium after which cells were harvested by centrifugation and resuspended at a density of 1 x 10⁷/ml. 100 μ l were added to each well of a

U-bottom microtiter plate containing 100 μ l per well of complete medium and diluted in 2-fold serial steps. Two wells for each sample did not contain lymphocytes, to allow spontaneous chromium release and total chromium uptake to be measured. The target cells, HeLa subline S3 (HeLa-S3, ATCC) were infected as above in 10 cm dishes with vPE16. 10^6 infected cells were detached with PBS and 1mM EDTA, centrifuged and resuspended in 100 μ l of 51 Cr sodium chromate (1 mCi/ml in PBS) for 1 hour at 37°C and then washed three times with PBS. 100 μ l of labelled target cells were added to each well. The microtiter plate was spun at 750 x g for 1 minute and incubated for 4 hours at 37°C. At the end of the incubation period, the cells in each well were resuspended by gentle pipetting, a sample removed to determine the total counts incorporated and the microtiter plate was spun at 750 x g for 1 min. Aliquots (100 μ l) of the supernatant were removed and counted in a gamma ray scintillation counter. The effector:target ratio was corrected for the percent of cells infected as measured by flow cytometry.

FIG. 22 shows replication of HIV-1 in transfectant cell lines. Cell lines stably expressing wild type CD4 and various recombinant chimeras were established in a subline of the human embryonal kidney cell line 293. A virus stock of the HIV-1 IIIB isolate was prepared with a titer of $\approx 10^6$ infectious particles/ml as measured by end-point dilution analysis using the human T-cell line C8166 as an indicator. Infections were carried out at an approximate MOI of 1 for a period of 8-12 hours at 37°C. On the following day the cells were washed with PBS three times, trypsinized, replated in new dishes and the culture supernatant sampled for p24 titer (designated day 0). At 3-4 day intervals thereafter, cell culture supernatants were collected and

retained for p24 analysis. The cells were resupplied with fresh medium containing hygromycin B at a concentration of 100 µg/ml. Analysis of culture supernatants was carried out using a commercial ELISA-based HIV-1 p24 antigen assay kit (Coulter) according to the instructions supplied by the manufacturer. Results are representative of two independent experiments of similar duration.

FIG. 23 shows the nucleic acid and amino acid sequence of the D1-D4 domains of CD4 (CD4 Bam).

FIG. 24 shows the nucleic acid and amino acid sequence of the D1-D2 domains of CD4 (CD4 Nhe).

FIG. 25 shows the nucleic acid and amino acid sequence of the hinge, CH2, and CH3 domains of human IgG1 (Igh23 Bam).

FIG. 26 shows the nucleic acid and amino acid sequence of the transmembrane domain of CD7 (TM7 Bam Mlu).

FIG. 27 shows the nucleic acid and amino acid sequence of the intracellular domain of zeta (Zeta Mlu Not).

FIG. 28 shows the DNA sequence and primary amino acid sequence of a synthetic alpha helix.

EXAMPLE I

Construction of Human IgG1:Receptor Chimeras

Human IgG1 heavy chain sequences were prepared by joining sequences in the C_H3 domain to a cDNA fragment derived from the 3' end of the transmembrane form of the antibody mRNA. The 3' end fragment was obtained by polymerase chain reaction using a tonsil cDNA library as substrate, and oligonucleotides having the sequences:

CGC GGG GTG ACC GTG CCC TCC AGC AGC TTG GGC (SEQ ID NO: 7) and

CGC GGG GAT CCG TCG TCC AGA GCC CGT CCA GCT CCC CGT CCT GGG CCT CA (SEQ ID NO: 8),

corresponding to the 5' and 3' ends of the desired DNA fragments respectively. The 5' oligo is complementary to a site in the C_H1 domain of human IgG1, and the 3' oligo is complementary to a site just 5' of the sequences encoding the membrane spanning domain. The PCR product was digested with BstXI and BamHI and ligated between BstXI and BamHI sites of a semisynthetic IgG1 antibody gene bearing variable and constant regions. Following the insertion of the BstXI to BamHI fragment, the amplified portions of the construct were replaced up to the SmaI site in C_H3 by restriction fragment interchange, so that only the portion between the SmaI site and the 3' oligo was derived from the PCR reaction.

To create a human IgG1:ζ chimeric receptor, the heavy chain gene ending in a BamHI site was joined to the BamHI site of the ζ chimera described below, so that the antibody sequences formed the extracellular portion. Flow cytometry of COS cells transfected with a plasmid encoding the chimera showed high level expression of antibody determinants when an expression plasmid encoding a light chain cDNA was cotransfected, and modest expression of antibody determinants when the light chain expression plasmid was absent.

Similar chimeras including human IgG1 fused to η or γ (see below), or any signal-transducing portion of a T cell receptor or Fc receptor protein may be constructed generally as described above using standard techniques of molecular biology.

To create a single transcription unit which would allow both heavy and light chains to be expressed from a single promoter, a plasmid encoding a bicistronic mRNA was created from heavy and light chain coding sequences, and the 5' untranslated portion of the mRNA encoding the 78kD

glucose regulated protein, otherwise known as grp78, or BiP. grp78 sequences were obtained by PCR of human genomic DNA using primers having the sequences:

CGC GGG CGG CCG CGA CGC CGG CCA AGA CAG CAC (SEQ ID
5 NO: 9) and

CGC GTT GAC GAG CAG CCA GTT GGG CAG CAG CAG (SEQ ID
NO: 10)

at the 5' and 3' ends respectively. Polymerase chain
reactions with these oligos were performed in the presence
10 of 10% dimethyl sulfoxide. The fragment obtained by PCR was
digested with NotI and HincII and inserted between NotI and
HpaI sites downstream from human IgG1 coding sequences.
Sequences encoding a human IgG kappa light chain cDNA were
then inserted downstream from the grp78 leader, using the
HincII site and another site in the vector. The expression
plasmid resulting from these manipulations consisted of the
semisynthetic heavy chain gene, followed by the grp78 leader
sequences, followed by the kappa light chain cDNA sequences,
followed by polyadenylation signals derived from an SV40 DNA
fragment. Transfection of COS cells with the expression
plasmid gave markedly improved expression of heavy chain
determinants, compared to transfection of plasmid encoding
heavy chain determinants alone.

To create a bicistronic gene comprising a heavy
25 chain/receptor chimera and a light chain, the upstream heavy
chain sequences can be replaced by any chimeric heavy chain/
receptor gene described herein.

EXAMPLE II

Construction of CD4 Receptor Chimeras

30 Human ζ (Weissman et al., Proc. Natl. Acad. Sci. USA
85:9709-9713 (1988b)) and γ (Küster et al., J. Biol. Chem.
265:6448-6452 (1990)) cDNAs were isolated by polymerase

chain reaction from libraries prepared from the HPB-ALL tumor cell line (Aruffo et al., Proc. Natl. Acad. Sci. USA 84:8573-8577 (1987b)) and from human natural killer cells, while η cDNA (Jin et al., Proc. Natl. Acad. Sci. USA

5 87:3319-3323 (1990)) was isolated from a murine thymocyte library. ζ , η and γ cDNAs were joined to the extracellular domain of an engineered form of CD4 possessing a BamHI site just upstream of the membrane spanning domain (Aruffo et al., Proc. Natl. Acad. Sci. USA 84:8573-8577 (1987b);

10 Zettlmeissl et al., DNA Cell Biol. 9:347-353 (1990)) which was joined to the BamHI site naturally present in the ζ and η cDNAs at a similar location a few residues upstream of the membrane spanning domain (SEQ ID NOS: 1, 3, 4 and 6). To form the fusion protein with γ a BamHI site was engineered into the sequence at the same approximate location (Fig. 1; SEQ ID NO: 2 and 5). The gene fusions were introduced into a vaccinia virus expression plasmid bearing the E. coli gpt gene as a selectable marker, and inserted into the genome of the vaccinia WR strain by homologous recombination and selection for growth in mycophenolic acid (Falkner et al., J. Virol. 62:1849-1854 (1988); Boyle et al., Gene 65:123-128 (1988)). Flow cytometric analysis showed that the vaccinia recombinants direct the abundant production of CD4: ζ and CD4: γ fusion proteins at the cell surface, whereas the

25 expression of CD4: η is substantially weaker (Fig. 1B). The latter finding is consistent with a recent report that transfection of an η cDNA expression plasmid into a murine hybridoma cell line gave substantially less expression than transfection of a comparable ζ expression plasmid (Clayton et al., J. Exp. Med. 172:1243-1253 (1990)).

30 Immunoprecipitation of cells infected with the vaccinia recombinants revealed that the fusion proteins form covalent dimers, unlike the naturally occurring CD4 antigen. The

099357.08401
FOR "280" 2E56650

molecular masses of the monomeric CD4:ζ and CD4:γ fusion proteins and native CD4 were found to be 63, 55 and 53 kD respectively. The larger masses of the fusion proteins are approximately consistent with the greater length of the intracellular portion, which exceeds that of native CD4 by 75 (CD4:ζ) or 5 (CD4:γ) residues.

EXAMPLE III

CD4 Chimeras Can Associate With Other Receptor Chains

Cell surface expression of the macrophage/natural killer cell form of human FcγRIII (CD16_{TM}) on transfectants is facilitated by cotransfection with murine (Kurosaki et al., Nature 342:805-807 (1989)) or human (Hibbs et al., Science 246:1608-1611 (1989)) γ, as well as by human ζ (Lanier et al., Nature 342:803-805 (1989)).

Consistent with these reports, expression of the chimeras also allowed surface expression of CD16_{TM} when delivered to the target cell either by cotransfection or by coinfection with recombinant vaccinia viruses (Fig. 2). The promotion of CD16_{TM} surface expression by ζ was more pronounced than promotion by γ (Fig. 2) in the cell lines examined, whereas native CD4 did not enhance CD16_{TM} surface expression.

EXAMPLE IV

Asp ζ Mutants Do Not Coassociate With Fc Receptor

To create chimeras which would not associate with existing antigen or Fc receptors, mutant ζ fusion proteins which lacked either the intramembranous Asp or intramembranous Cys residue or both were prepared. Flow cytometry showed that the intensity of cell surface expression by the different mutant chimeras was not appreciably different from the unmutated precursor, and

immunoprecipitation experiments showed that total expression by the chimeras was similar. As expected, the mutant chimeras lacking the transmembrane cysteine residue were found not to form disulfide linked dimers. The two mutant chimeras lacking Asp were incapable of supporting the surface expression of CD16TM, whereas the monomeric chimeras lacking Cys but bearing Asp allowed CD16TM to be coexpressed, but at lower efficiency than the parental dimer (Fig. 3).

EXAMPLE V

Mutant Receptors Retain the Ability to Initiate a Calcium Response

To determine whether crosslinking of the fusion proteins would allow the accumulation of free intracellular calcium in a manner similar to that known to occur with the T cell antigen receptor, cells of the human T cell leukemia line, Jurkat E6 (ATCC Accession Number TIB 152, American Type Culture Collection, Rockville, MD), were infected with the vaccinia recombinants and the relative cytoplasmic calcium concentration following crosslinking of the extracellular domain with antibodies was measured. Flow cytometric measurements were performed with cells loaded with the calcium sensitive dye Indo-1 (Grynkiewicz et al., J. Biol. Chem. 260:3340-3450 (1985); Rabinovitch et al., J. Immunol. 137:952-961 (1986)). Figure 4A-D shows the results of calcium flux experiments with cells infected with CD4:ζ and the Asp⁻ and Cys⁻ mutants of ζ. Crosslinking of the chimeras, reproducibly increased intracellular calcium. CD4:η and CD4:γ similarly allowed accumulation intracellular calcium in infected cells. Jurkat cells express low levels of CD4 on the cell surface, however, crosslinking of the

native CD4 in the presence or absence of CD16:ζ does not alter intracellular calcium levels (Fig. 4A-B).

EXAMPLE VI

CD4:ζ, η, and γ Chimeras Mediate Cytolysis of Targets Expressing HIV gp120/41

To determine whether the chimeric receptors would trigger cytolytic effector programs, a model target:effector system based on CD4 recognition of the HIV envelope gp120/gp41 complex was created. HeLa cells were infected with recombinant vaccinia viruses expressing gp120/gp41 (Chakrabarti et al., Nature 320:535-537 (1986); Earl et al., J. Virol. 64:2448-2451 (1990)) and labeled with ⁵¹Cr. The labeled cells were incubated with cells from a human allospecific (CD8⁺, CD4⁻) cytotoxic T lymphocyte line which had been infected with vaccinia recombinants expressing the CD4:ζ, CD4:η, or CD4:γ chimeras, or the CD4:ζCys11Gly:Asp15Gly double mutant chimera. Fig. 5A-C shows that HeLa cells expressing gp120/41 were specifically lysed by cytotoxic T lymphocytes (CTL) expressing CD4 chimeras. Uninfected HeLa cells were not targeted by CTL armed with CD4:ζ chimeras, and HeLa cells expressing gp120/41 were not recognized by uninfected CTL. To compare the efficacy of the various chimeras, the effector to target ratios were corrected for the fraction of CTL expressing CD4 chimeras, and for the fraction of HeLa cells expressing gp120/41, as measured by flow cytometry. Fig. 5C shows a cytometric analysis of CD4 expression by the CTL used in the cytolysis experiment shown in Figs. 5A and 5B. Although the mean density of surface CD4:ζ greatly exceeded the mean density of CD4:η, the cytolytic efficiencies of cells expressing either form were similar. Correcting for the fraction of targets expressing gp120, the efficiency of

cytolysis mediated by CD4:ζ and CD4:η proteins are comparable to the best efficiencies reported for specific T cell receptor target:effector pairs (the mean effector to target ratio for 50% release by T cells expressing CD4:ζ was 1.9 ± 0.99, n=10. The CD4:γ fusion was less active, as was the CD4:ζ fusion lacking the transmembrane Asp and Cys residues. However in both cases significant cytolysis was observed (Fig. 5B-C).

To control for the possibility that vaccinia infection might promote artefactual recognition by CTL, similar cytolysis experiments were performed with target cells infected with vaccinia recombinants expressing the phosphatidylinositol linked form of CD16 (CD16_{PI}) and labeled with ⁵¹Cr, and with CTL infected with control recombinants expressing either CD16_{PI} or CD16:ζ. Fig. 6A shows that T cells expressing non-CD4 chimeras do not recognize native HeLa cells or HeLa cells expressing gp120/41, and similarly that T cells expressing CD4 chimeras do not recognize HeLa cells expressing other vaccinia-encoded surface proteins. In addition, CTLs expressing non-chimeric CD4 do not significantly lyse HeLa cells expressing gp120/41 (Fig. 6A).

EXAMPLE VII

MHC Class II-Bearing Cells Are Not Targeted by the Chimeras

CD4 is thought to interact with a nonpolymorphic sequence expressed by MHC class II antigen (Gay et al., Nature 328:626-629 (1987); Sleckman et al., Nature 328:351-353 (1987)). Although a specific interaction between CD4 and class II antigen has never been documented with purified proteins, under certain conditions adhesion between cells expressing CD4 and cells expressing class II molecules can be demonstrated (Doyle et al., Nature 330:256-259 (1987);

Clayton et al., J. Exp. Med. 172:1243-1253 (1990); Lamarre
et al., Science 245:743-746 (1989)). Next examined was
whether killing could be detected against cells bearing
class II. Fig. 6B shows that there is no specific cytotoxicity
directed by CD4:ζ against the Raji B cell line, which
expresses abundant class II antigen. Although a modest
(≈5%) cytotoxicity is observed, a class II-negative mutant of
Raji, RJ2.2.5, (Accolla, J. Exp. Med. 157:1053-1058 (1983))
shows a similar susceptibility, as do Raji cells incubated
with uninfected T cells.

EXAMPLE VIII

Sequence Requirements for Induction of Cytotoxicity by the T Cell Antigen/Fc Receptor Zeta Chain

Although chimeras between CD4 and ζ can arm
cytotoxic T lymphocytes (CTL) to kill target cells
expressing HIV gp120, an alternative to CD4 was sought in
order to unambiguously compare the properties of zeta
chimeras introduced into human T cell lines. Such lines can
express CD4, making it difficult to specifically define the
relationship between the type or degree of calcium
mobilization and the cytotoxic potential of the different
chimeras. To circumvent this, chimeras were created between
ζ and CD16 in which the extracellular domain of CD16 is
attached to the transmembrane and intracellular sequences of
ζ (Fig. 7A). The gene fusions were introduced into a
vaccinia virus expression plasmid bearing the E. coli gpt
gene as a selectable marker and inserted into the genome of
the vaccinia WR strain by homologous recombination and
selection for growth in mycophenolic acid (Falkner and Moss,
J. Virol. 62:1849 (1988); Boyle and Coupar, Gene 65:123
(1988)).

T cell lines were infected with the vaccinia recombinants and the relative cytoplasmic free calcium ion concentration was measured following crosslinking of the extracellular domains with antibodies. Both spectrofluorimetric (bulk population) and flow cytometric (single cell) measurements were performed with cells loaded with the dye Indo-1 (Gryniewicz et al., J. Biol. Chem. 260:3440 (1985); Rabinovitch et al., J. Immunol. 137:952 (1986)). Figure 7B shows an analysis of data collected from cells of the Jurkat human T cell leukemia line infected with vaccinia recombinants expressing CD16:ζ fusion protein. Crosslinking of the chimeras reproducibly increased intracellular calcium, while similar treatment of cells expressing nonchimeric CD16 had little or no effect. When the chimera was expressed in mutant cell lines lacking antigen receptor, either REX33A (Breitmeyer et al., J. Immunol. 138:726 (1987); Sancho et al., J. Biol. Chem. 264:20760 (1989)), or Jurkat mutant JRT3.T3.5 (Weiss et al., J. Immunol. 135:123 (1984)); or a strong response to CD16 antibody crosslinking was seen. Similar data have been collected on the REX20A (Breitmeyer et al., supra, 1987; Blumberg et al., J. Biol. Chem. 265:14036 (1990)) mutant cell line, and a CD3/Ti negative mutant of the Jurkat cell line established in this laboratory. Infection with recombinants expressing CD16:ζ did not restore the response to anti-CD3 antibody, showing that the fusion protein did not act by rescuing intracellular CD3 complex chains.

To evaluate the ability of the chimeras to redirect cell-mediated immunity, CTLs were infected with vaccinia recombinants expressing CD16 chimeras and used to specifically lyse hybridoma cells expressing membrane-bound anti-CD16 antibodies. This assay is an extension of a hybridoma cytotoxicity assay originally developed to analyze

effector mechanisms of cells bearing Fc receptors (Graziano and Fanger, J. Immunol. 138:945, 1987; Graziano and Fanger, J. Immunol. 139:35-36, 1987; Shen et al., Mol. Immunol. 26:959, 1989; Fanger et al., Immunol. Today 10: 92, 1989).

5 Fig. 8B shows that expression of CD16:ζ in cytotoxic T lymphocytes allows the armed CTL to kill 3G8 (anti-CD16; Fleit et al., Proc. Natl. Acad. Sci. USA 79:3275, 1982) hybridoma cells, whereas CTL expressing the phosphatidylinositol-linked form of CD16 are inactive. CTL
10 armed with CD16:ζ also do not kill hybridoma cells expressing an irrelevant antibody.

Sub
Ag
To identify the minimal ζ sequences necessary for
cytolysis, a series of deletion mutants were prepared in
which successively more of the ζ intracellular domain was
15 removed from the carboxyl terminus (Fig. 8A). Most of the intracellular domain of zeta could be removed with little consequence for cytolytic potential; the full length chimera CD16:ζ was essentially equal in efficacy to the chimera deleted to residue 65, CD16:ζAsp66* (Fig. 8B). A
20 substantial decrease in cytotoxicity was observed on deletion to ζ residue 59 (chimera CD16:ζGlu60*), and further deletion to residue 50 resulted in slightly less activity. However complete loss of activity was not observed even when the intracellular domain was reduced to a three residue
25 transmembrane anchor (Fig. 8B).

Because ζ is a disulfide linked dimer, one explanation for the retention of cytolytic activity was that endogenous ζ was forming heterodimers with the chimeric ζ deletion, thereby reconstituting activity. To test this
30 idea, ζ residues 11 and 15 were changed from Asp and Cys respectively to Gly (Cys11Gly/Asp15Gly), and immunoprecipitations were carried out as follows. Approximately 2×10^6 CV1 cells were infected for one hour

in serum free DME medium with recombinant vaccinia at a multiplicity of infection (moi) of at least ten. Six to eight hours post-infection, the cells were detached from the plates with PBS/1mM EDTA and surface labeled with 0.2 mCi ^{125}I per 2×10^6 cells using lactoperoxidase and H_2O_2 by the method of Clark and Einfeld (*Leukocyte Typing II*, pp 155-167, Springer-Verlag, NY, 1986). The labeled cells were collected by centrifugation and lysed in 1% NP-40, 0.1% SDS, 0.15M NaCl, 0.05M Tris, pH 8.0, 5mM MgCl_2 , 5mM KCl, 0.2M iodoacetamide and 1mM PMSF. Nuclei were removed by centrifugation, and CD16 proteins were immunoprecipitated with antibody 3G8 (Fleit et al., *supra*, 1982; Medarex) and anti-mouse IgG agarose (Cappel, Durham, NC). Samples were electrophoresed through an 8% polyacrylamide/SDS gel under non-reducing conditions or through a 10% gel under reducing conditions. These immunoprecipitations confirmed that the CD16: ζ Cys11Gly/Asp15Gly chimera did not associate in disulfide-linked dimer structures.

The cytolytic activity of the mutant receptors was also tested. The mutated chimera deleted to residue 65 (CD16: ζ Cys11Gly/Asp15Gly/Asp66*) was, depending on the conditions of assay, two to eight fold less active in the cytotoxicity assay than the comparable unmutated chimera (CD16: ζ Asp66*), which was usually within a factor of two of, or indistinguishable in activity from, CD16: ζ (Fig. 9B). The reduction in activity of the mutant chimeras is comparable to the reduction seen with CD4 chimeras of similar structure (see above) and is most likely attributable to the lower efficiency of ζ monomers compared to dimers. In contrast, the Asp⁻, Cys⁻ mutated chimera deleted to residue 59 had no cytolytic activity (Fig. 9B), supporting the hypothesis that association with other chains mediated by the transmembrane Cys and/or Asp residues was

responsible for the weak persistence of cytolytic activity in deletions more amino terminal than residue 65.

Flow cytometric studies showed that the deletion mutants lacking transmembrane Asp and Cys residues could still promote an increase in free intracellular calcium ion in response to antibody crosslinking in a TCR⁻ mutant Jurkat cell line (Fig. 9D). Similar results were obtained for chimeras expressed in the parental Jurkat line. In the case of CD16:ζCys11Gly/Asp15Gly/Glu60*, these data demonstrate that the ability to mediate calcium responsiveness can be mutationally separated from the ability to support cytolysis.

To definitively eliminate the possible contribution of ζ transmembrane residues, the transmembrane and first 17 cytoplasmic residues of ζ were replaced by sequences encoding the membrane spanning and first 14 or first 17 cytoplasmic residues of the CD5 or CD7 antigens, respectively (Fig. 9A). The resulting tripartite fusion proteins CD16:5:ζ(48-65) and CD16:7:ζ(48-65) did not form disulfide-linked dimers as do the simpler CD16:ζ chimeras, because they lacked the cysteine residue in the ζ transmembrane domain. Both tripartite chimeras were able to mobilize calcium in Jurkat and TCR negative cell lines (Fig. 9D) and to mount a cytolytic response in CTL (Fig. 9C and data not shown). However truncation of the ζ portion to residue 59 in chimera CD16:7:ζ(48-59) abrogates the ability of tripartite fusion to direct calcium responsiveness in TCR positive or negative Jurkat cells or cytolysis in mature CTL (Fig. 9C and 9D and data not shown).

To examine the contributions of individual residues within the 18-residue motif, we prepared a number of mutant variants by site-directed mutagenesis, and evaluated their ability to mediate receptor-directed killing under

conditions of low (Figs. 10A and 10D) or high (Figs. 10B and 10E) expression of chimeric receptor. Fig. 10A-F shows that while a number of relatively conservative substitutions (i.e., replacing acidic residues with their cognate amides, or tyrosine with phenylalanine) which spanned residues 59 to 63 yielded moderate compromise of cytolytic efficacy, in general the variants retained the ability to mobilize calcium. However collectively these residues comprise an important submotif inasmuch as their deletion eliminates cytolytic activity. Conversion of Tyr 62 to either Phe or Ser eliminated both the cytotoxic and calcium responses. At the amino terminus of the 18 residue segment, replacement of Tyr 51 with Phe abolished both calcium mobilization and cytolytic activity, while substitution of Leu with Ser at position 50 eliminated the calcium response while only partially impairing cytolysis. Without being bound to a particular hypothesis, it is suspected that the inability of the Leu50Ser mutant to mobilize calcium in short term flow cytometric assays does not fully reflect its ability to mediate a substantial increase in free intracellular calcium ion over the longer time span of the cytolysis assay. However, calcium-insensitive cytolytic activity has been reported for some cytolytic T cell lines, and the possibility that a similar phenomenon underlies the results described herein has not been ruled out. Replacement of Asn48 with Ser partially impaired cytotoxicity in some experiments while having little effect in others.

To investigate the potential role of redundant sequence elements, the intracellular domain of ζ was divided into three segments, spanning residues 33 to 65, 71 to 104, and 104 to 138. Each of these segments was attached to a CD16:CD7 chimera by means of a MluI site introduced just distal to the basic membrane anchoring sequences of the

intracellular domain of CD7 (see below; Fig. 11A).
Comparison of the cytolytic efficacy of the three elements
showed they were essentially equipotent (Fig. 11B).
Sequence comparison (Fig. 11A) shows that the second motif
5 bears eleven residues between tyrosines, whereas the first
and third motifs bear ten.

Although a precise accounting of the process of T
cell activation has not been made, it is clear that
aggregation of the antigen receptor, or of receptor chimeras
10 which bear ζ intracellular sequences, triggers calcium
mobilization, cytokine and granule release, and the
appearance of cell surface markers of activation. The
active site of ζ , a short linear peptide sequence probably
too small to have inherent enzymatic activity, likely
interacts with one or at most a few proteins to mediate
cellular activation. It is also clear that mobilization of
free calcium is not by itself sufficient for cellular
activation, as the ability to mediate cytotoxicity can be
mutationally separated from the ability to mediate calcium
accumulation.

As shown herein, addition of 18 residues from the
intracellular domain of ζ to the transmembrane and
intracellular domain of two unrelated proteins allows the
resulting chimeras to redirect cytotoxic activity against
25 target cells which bind to the extracellular portion of the
fusion proteins. Although chimeras bearing the 18 residue
motif are approximately eight-fold less active than chimeras
based on full length ζ , the reduced activity can be
attributed to the loss of transmembrane interactions which
30 normally allow wild-type ζ to form disulfide linked dimers.
That is, ζ deletion constructs which have the same carboxyl
terminus as the motif and lack transmembrane Cys and Asp

residues typically show slightly less activity than chimeras bearing only the 18 residue motif.

5 The cytolytic competency element on which we have focused has two tyrosines and no serines or threonines, restricting the possible contributions of phosphorylation to activity. Mutation of either tyrosine destroys activity, however, and although preliminary experiments do not point to a substantial tyrosine phosphorylation following crosslinking of chimeric surface antigens bearing the 18
10 residue motif, the possible participation of such phosphorylation at a low level cannot be excluded. In addition to the effects noted at the two tyrosine residues, a number of amino acid replacements at the amino and carboxyl terminus of the motif weaken activity under
5 conditions of low receptor density.

Sequences similar to the ζ active motif can be found in the cytoplasmic domains of several other transmembrane proteins, including the CD3 δ and γ molecules, the surface IgM associated proteins mb1 and B29, and the β and γ chains
20 of the high affinity IgE receptor, Fc ϵ RI (Reth, Nature 338:383, 1989). Although the function of these sequences is uncertain, if efficiently expressed, each may be capable of autonomous T cell activation, and such activity may explain the residual TCR responsiveness seen in a zeta-negative
25 mutant cell line (Sussman et al., Cell 52:85, 1988).

ζ itself bears three such sequences, approximately equally spaced, and a rough trisection of the intracellular domain shows that each is capable of initiating a cytolytic response. η , a splice isoform of ζ (Jin et al., supra,
30 1990; Clayton et al., Proc. Natl. Acad. Sci. USA 88:5202, 1991), lacks the carboxyl half of the third motif. Because removal of the carboxyl half of the first motif abolishes activity, it appears likely that the majority of the

05937537 082404
FOI-280 2E56E5D

biological effectiveness of η can be attributed to the first two motifs. Although by different measures η is equally as active as ζ in promoting antigen-mediated cytokine release (Bauer et al., Proc. Natl. Acad. Sci. USA 88:3842, 1991) or redirected cytolysis (see above), η is not phosphorylated in response to receptor stimulation (Bauer et al., supra, 1991). Thus either the presence of all three motifs is required for phosphorylation, or the third motif represents a favored substrate for an unidentified tyrosine kinase.

EXAMPLE IX

Cytolytic Signal Transduction by Human Fc Receptor

To evaluate the actions of different human Fc receptor subtypes, chimeric molecules were created in which the extracellular domain of the human CD4, CD5 or CD16 antigens were joined to the transmembrane and intracellular domains of the FcRIIA, B1, B2, and C subtypes (nomenclature of Ravetch and Kinet, Ann. Rev. Immunol. 9:457, 1991). Specifically, cDNA sequences corresponding to the transmembrane and cytoplasmic domains of the previously described FcRIIA, B1, and B2 isoforms were amplified from the preexisting clone PC23 or from a human tonsil cDNA library (constructed by standard techniques) using the following synthetic oligonucleotides primers:

CCC GGA TCC CAG CAT GGG CAG CTC TT (SEQ ID NO: 18;
FcRII A forward);

CGC GGG GCG GCC GCT TTA GTT ATT ACT GTT GAC ATG GTC
GTT (SEQ ID NO: 19; FcRII A reverse);

GCG GGG GGA TCC CAC TGT CCA AGC TCC CAG CTC TTC ACC
G (SEQ ID NO: 20; FcRII B1 and FcRII B2 forward); and

GCG GGG GCG GCC GCC TAA ATA CGG TTC TGG TC (SEQ ID
NO: 21; FcRII B1 and FcRII B2 reverse).

These primers contained cleavage sites for the enzymes BamHI and NotI, respectively, indented 6 residues from the 5' end. The NotI site was immediately followed by an antisense stop codon, either CTA or TTA. All primers contained 18 or more residues complementary to the 5' and 3' ends of the desired fragments. The cDNA fragment corresponding to the FcRII γ C cytoplasmic domain, which differs from the IIA isoform in only one amino acid residue (L for P at residue 268) was generated by site directed mutagenesis by overlap PCR using primers of sequence:

TCA GAA AGA GAC AAC CTG AAG AAA CCA ACA A (SEQ ID NO:22) and

TTG TTG GTT TCT TCA GGT TGT GTC TTT CTG A (SEQ ID NO: 23).

The PCR fragments were inserted into vaccinia virus expression vectors which contained the CD16 or CD4 extracellular domains respectively and subsequently inserted into wild type vaccinia by recombination at the thymidine kinase locus, using selection for cointegration of E coli gpt to facilitate identification of the desired recombinants. The identities of all isoforms (shown in Fig. 12) were confirmed by dideoxy sequencing.

Production of the chimeric receptor proteins was further confirmed by immunoprecipitation studies.

Approximately 10^7 JRT3.T3.5 cells were infected for one hour in serum free IMDM medium with recombinant vaccinia at a multiplicity of infection of at least ten. Twelve hours post-infection, the cells were harvested and surface labeled with 0.5mCi ^{125}I per 10^7 cells using the

lactoperoxidase/glucose oxidase method (Clark and Einfeld, supra). The labeled cells were collected by centrifugation and lysed 1% NP-40, 0.1mM MgCl_2 , 5mM KCl, 0.2M iodoacetamide and 1mM PMSF. Nuclei were removed by centrifugation, and

CD16 fusion proteins immunoprecipitated with antibody 4G8 and anti-mouse IgG agarose. Samples were electrophoresed under reducing conditions. All immunoprecipitated chimeric receptor molecules were of the expected molecular masses.

5 To test the ability of the chimeric receptors to mediate an increase in cytoplasmic free calcium ion, the recombinant viruses were used to infect the TCR⁻ mutant Jurkat cell line JRT3.T3.5 (as described herein) and cytoplasmic free calcium was measured in the cells (as
10 described herein) following crosslinking of the receptor extracellular domains with monoclonal antibody 3G8 or Leu-3A (as described herein). These experiments revealed that the intracellular domains of FcR γ II A and C were capable of mediating an increase in cytoplasmic free calcium ion after crosslinking of the extracellular domains, whereas the intracellular domains of FcR γ II B1 and B2 were inactive under comparable conditions (Fig. 13 A and 13B). The CD4, CD5 and CD16 hybrids of FcR γ II A shared essentially equal capacity to promote the calcium response (Fig. 13A-B).
15 Other cell lines, from both monocytic and lymphocytic lineages, were capable of responding to the signal initiated by crosslinking of the extracellular domains.

To explore the involvement of the different FcR γ II intracellular domains in cytotoxicity, human cytotoxic T
25 lymphocytes (CTL) were infected with vaccinia recombinants expressing CD16:FcR γ II A, B1, B2 and C chimeras. The infected cells were then cocultured with ⁵¹Cr-loaded hybridoma cells (i.e., 3G8 10-2 cells) which expressed cell surface antibody to CD16. In this assay CTLs bearing the
30 CD16 chimera killed the hybridoma target cells (allowing release of free ⁵¹Cr) if the CD16 extracellular domain of the chimera has been joined to an intracellular segment capable of activating the lymphocyte effector program; this

cytolysis assay is described in detail below. Fig. 14A shows that CTL armed with CD16:FcRyIIA and C, but not FcRyII B1 or B2, are capable of lysing target cells expressing cell surface anti-CD16 antibody.

5 To eliminate the possibility that the specific cytolysis was in some way attributable to interaction with the CD16 moiety, cytolysis experiments were conducted in which the FcRII intracellular domains were attached to a CD4 extracellular domain. In this case the target cells were
10 HeLa cells expressing HIV envelope gp120/41 proteins (specifically, HeLa cells infected with the vaccinia vector vPE16 (available from the National Institute of Allergy and Infections Disease AIDS Depository, Bethesda, MD). As in the CD16 system, target cells expressing HIV envelope were susceptible to lysis by T cells expressing the CD4:FcRyII A chimera, but not FcRyII B1 or B2 (Fig. 14B).

FOI 200-255650
10420
The intracellular domains of FcRyII A and C share no appreciable sequence homology with any other protein, including the members of the extended FcRy/TCR ζ family. To define the sequence elements responsible for induction of cytolysis, 5' and 3' deletions of the intracellular domain coding sequences (described below and shown in Fig. 15A) were prepared and were evaluated for efficacy in calcium mobilization and cytolysis assays (as described herein). In
25 the experiments in which the amino terminal portion of the intracellular domain was removed, the transmembrane domain of FcRyII was replaced with the transmembrane domain of the unrelated CD7 antigen to eliminate the possible contribution of interactions mediated by the membrane-spanning domain.

30 Figs. 15B and 15C show that removal of the 14 carboxyl-terminal residues, including tyrosine 298, resulted in a complete loss of cytolytic capacity and a substantial reduction in calcium mobilization potential. Further

deletion to just before tyrosine 282 gave an identical phenotype (Figs. 15B and 15C). Deletion from the N-terminus of the intracellular domain to residue 268 had no substantial effect on either calcium profile or cytolytic potency, whereas deletion to residue 275 markedly impaired free calcium release but had little effect on cytolysis (Figs. 15D and 15E). Further deletion, to residue 282, gave FcRyII tails which lacked the ability to either mobilize calcium or trigger cytolysis (Figs. 15D and 15E). The 'active element' defined by these crude measures is relatively large (36 amino acids) and contains two tyrosines separated by 16 residues.

EXAMPLE X

Targeted Cytolysis by Lymphocytes Bearing Chimeric CD4 Receptors Which Do Not Support Infection

As discussed above, effector molecules may be engineered which redirect the cytolytic activity of CTLs in an MHC-independent manner. For example, a chimera composed of the extracellular domain of CD4 fused to the ζ chain in a human CTL clone, WH3, specifically kills target cells displaying the surface envelope glycoprotein of HIV-1, gp120. Since the extracellular domain of the CD4 molecule confers susceptibility to HIV infection, however, the armed CTLs may become targets for the virus, resulting in a decrease in their potency (Dalglish et al., Nature 312:767 (1984); Klatzmann et al., Nature 312:767 (1984)). To prevent such an outcome, chimeric effector molecules were designed based on CD4 which are effective in specifically targeting HIV-infected cells for cell-mediated killing but which do not confer susceptibility to infection by HIV.

A tripartite fusion protein was created by genetic apposition of the extracellular domain of CD4 (Fig. 23) to

the hinge, second, and third constant domains of human IgG1 heavy chain (Zettlmeissl et al. DNA Cell Biol. 9:347 (1990)) (Fig. 25), which were joined in this case to a portion of the first transmembrane exon of human membrane-bound IgG1, followed by a portion of the human CD7 antigen consisting of the sequences between the sole Ig-like domain and the stop transfer sequence following the transmembrane domain (Aruffo and Seed, EMBO J. 6:3313 (1987)) (Fig. 26). The primary amino acid sequence of the extracellular moiety of the CD7 segment consisted of a proline-rich region suggestive of a stalk-like structure which projects the Ig-like domain away from the cell surface (Aruffo and Seed EMBO J. 6:3313 (1987)) (Fig. 26). Recombinant vaccinia viruses were prepared to express this and related chimeras as described herein. In particular, recombinant vaccinia viruses were generated by homologous recombination in CV-1 cells. At least two rounds of plaque visualization with OKT4 or Leu3a followed by plaque purification was performed for each stock prior to preparation of high titer stocks in CV-1 cells.

The tripartite chimera ((CD4:(D1-D4):Ig:CD7)) (Fig. 20, molecule "A") showed efficient cell surface expression and was tested for the ability to act as an HIV receptor in a vaccinia-based syncytia formation assay (Lifson et al., Nature 323:725 (1986)); Ashorn et al., J. Virol. 64:2149 (1990)). HeLa cells infected with a recombinant vaccinia virus (vPE16) encoding the envelope glycoprotein of HIV-1 (Earl et al., J. Virol. 64:2448 (1990)) were co-cultured with HeLa cells infected either with CD4, CD4:ζ, or CD4(D1-D4):Ig:CD7. Six cm dishes of HeLa cells (ATCC, Rockville, MD) at 50% confluence were infected in serum-free medium for 1 hour at an approximate multiplicity of infection (MOI) of 10. The cells were incubated for an additional 5-6 hours in complete medium and then detached with phosphate buffered

saline (PBS) containing 1mM EDTA. Cells expressing envelope and CD4 chimera were mixed at a 1:1 ratio, and replated in 6 cm dishes with complete medium. Syncytia were scored at 6-8 hours post-cocultivation and photographed.

5 Co-cultures of CD4 and vPE16 led to formation of readily detectable multinucleated giant cells. Also, a chimera consisting of the extracellular domain of CD4 fused to the ζ chain of the TCR (Fig. 27) (CD4: ζ) was able to mediate syncytia formation, whereas cells expressing CD4(D1-D4):Ig:CD7 gave no sign of cell fusion. We also tested a construct expressing only the first and second domains of CD4 (Fig. 24), CD4(D1,D2):Ig:CD7 (Fig. 20, molecule "B"), since in another context the amino terminal two domains of the CD4 have been shown to be necessary for infectivity by HIV (Landau et al., Nature 334:159 (1988)). This molecule proved insusceptible to HIV-induced syncytia formation as well. Binding studies with soluble ^{125}I -labelled gp120 established that both CD4(D1-D4):Ig:CD7 and CD4(D1,D2):Ig:CD7 had uncompromised affinity for gp120.

10 We next determined whether chimeric molecules which resisted syncytium formation would be able to redirect cell killing if endowed with a trigger moiety as described herein. We fused the intracellular domain of ζ (Fig. 27) to the 3' end of CD4(D1-D4):Ig:CD7 and CD4(D1,D2):Ig:CD7 and prepared the corresponding recombinant vaccinia viruses. These constructs, CD4(D1-D4):Ig:CD7: ζ and CD4(D1,D2):Ig:CD7: ζ (Fig. 20, molecules "C" and "D"), were expressed in the human CTL clone WH3 and tested for their ability to target and kill HeLa cells expressing the surface envelope glycoprotein of HIV (using the methods described herein). Fig. 21 shows that the intracellular domain of ζ fused to either CD4(D1-D4):Ig:CD7 or CD4(D1,D2):Ig:CD7 can confer killing ability; constructs lacking the ζ chain were

not able to mediate this activity. CD4:ζ, a positive control, mediated a slightly more effective cytotoxicity, and CD4(D1,D2):Ig:CD7:ζ a somewhat less effective cytotoxicity than CD4(D1-D4):Ig:CD7:ζ (Fig. 21). However, it is clear that both CD4(D1-D4):Ig:CD7:ζ and CD4(D1,D2):Ig:CD7:ζ chimeras have the capacity to mediate specific killing of cells expressing HIV envelope proteins on their surface. The tetrapartite chimeras were consistently incapable of mediating syncytium formation in the vaccinia-based assay. We have also demonstrated that a single ζ motif of the sort shown in Fig. 11A is sufficient to confer cytolytic activity to a CD4(D1-D4) chimera.

Radioimmunoprecipitation experiments established that the fusion molecules were predominantly if not entirely dimers. In these experiments, protein-A agarose beads were used to immunoprecipitate the solubilized extract of metabolically labelled HeLa cells infected with recombinant vaccinia expressing CD4(D1-D4):Ig:CD7:ζ and CD4(D1,D2):Ig:CD7:ζ chimeras. The immunoprecipitated material was fractionated by polyacrylamide gel electrophoresis under reducing and nonreducing conditions. In particular, approximately 5×10^6 HeLa-S3 cells were infected as described above for vPE16 with the appropriate vaccinia virus stock. Cells were metabolically labelled with 200 μCi/ml of Tran³⁵S-Label (ICN Radiochemicals, Irvine, CA) for 6-8 hours in cysteine and methionine-deficient medium and detached with PBS containing 1mM EDTA. Cells were subsequently pelleted and lysed in 150mM NaCl, 50mM Tris pH 7.5, 5mM EDTA, 0.5% NP-40, 0.1% SDS, 5mM EDTA, 1mM PMSF. Following the removal of the nuclei by centrifugation, one fifth of each cell extract was adsorbed onto washed protein A-conjugated agarose beads for 2 hours at 4°C. The beads were subsequently washed with PBS

containing 1% NP-40 and eluted in sample buffer containing SDS in the presence or absence of mercaptoethanol. The results of these experiments demonstrated that the majority of the immunoprecipitated CD4(D1-D4):Ig:CD7:ζ and
5 CD4(D1,D2):Ig:CD7:ζ chimeras migrated as dimers of the expected molecular mass under nonreducing conditions.

To directly evaluate the ability of cells expressing the CD4 fusion molecules to support HIV infection, we performed long term infectivity studies on transfectants
10 expressing CD4(D1-D4):Ig:CD7 and CD4(D1,D2):Ig:CD7. Stable transfectants of CD4(D1-D4):Ig:CD7 and CD4(D1,D2):Ig:CD7 and CD4 were prepared in a subline of 293 cells, a readily transfectable cell line of human embryonic kidney origin. The chimeric molecules were subcloned in bidirectional
15 vectors in which the hygromycin B gene was driven by the herpes simplex virus thymidine kinase promoter. A 60-70% confluent 10 cm dish of cells was transfected with 10 μg of this plasmid DNA by calcium phosphate coprecipitation. Prior to transfection, the plasmids were linearized at the
20 unique Sfi I site, and the ends made flush with T4 DNA polymerase. At 24 hours post-transfection, the cells were split fourfold and at 48 hours post-transfection the cells were put under selection with hygromycin B (Sigma, St. Louis, Mo) at 400 μg/ml. Every 3-4 days, cells were
25 supplied with fresh medium containing hygromycin.

Resistant colonies were picked, expanded, and their expression assessed by indirect immunofluorescence using fluorescein-conjugated anti-human IgG Fc (Organon Teknika, West Chester, PA) or Q4120, an antibody reactive with human
30 CD4 (Sigma) followed by flow cytometry (Coulter, Hialeah, FL). Two independent clones from each construct with levels of cell surface CD4 comparable to that shown by the other cell lines were selected for analysis. Fig. 22 shows that,

following exposure to HIV, p24 was detected in the CD4 stable transfectant cultures as early as 3 days post-infection. The presence of multinucleated giant cells and characteristic ballooning was evident as early as 5 days post-infection in these cultures. No significant p24 levels or evidence of multinucleated giant cells was detectable in the untransfected parental cell line or in either of two independently derived isolates of CD4(D1-D4):Ig:CD7 and CD4(D1,D2):Ig:CD7 transfectants after 32 days in culture (Fig. 22).

Upon completion of the infectivity studies, cells were analyzed for cell surface CD4 expression. CD4 surface epitope density was significantly reduced in infected cultures expressing CD4, consistent with viral down-modulation, but was unaffected in cultures expressing CD4(D1-D4):Ig:CD7 and CD4(D1,D2):Ig:CD7. These experiments establish that it is possible to create chimeric molecules bearing the apical two domains of CD4 which, when fused to T cell receptor ζ chain, have the capacity to target and kill HIV-infected cells, but which do not support CD4-mediated HIV infection.

Additional experiments suggest that it is the physical distance between the extracellular domain of the CD4 molecule and the lipid bilayer that confers the ability to resist HIV infection. In a first experiment, we constructed a chimeric molecule bearing a deletion of the CD7 stalk and transmembrane domain; this deletion removed the proline rich region of the CD7 transmembrane portion. When this domain was fused to the extracellular domain of CD4, it maintained its ability to efficiently anchor the extracellular domain of the CD4 molecule, as measured by cell surface expression of the CD4 molecule (as described herein). However, the potential to resist syncytium

formation induced by the HIV envelope glycoprotein was lost. Thus, deletion of the proline-rich region of the CD7 molecule, a region likely to form an α -helical coil structure, effectively reduced the distance between the extracellular domain of CD4 and the lipid bilayer and abrogated the ability of the chimera to resist syncytium formation.

In a second experiment, we demonstrated that the ability to resist HIV-induced syncytium formation may be conferred upon a CD4/CD5 chimera which had previously been documented to serve as a transmembrane anchor for a CD4 extracellular domain but which was unable to resist HIV-induced syncytium formation. In this experiment, the hinge, CH2, and CH3 domains of the human IgG1 heavy chain were inserted into the CD4/CD5 molecule; the resulting chimera resisted syncytium formation, again suggesting that the distance afforded by the immunoglobulin domains is sufficient to confer resistance to HIV-induced syncytium formation.

In a third experiment, a CD4 domain was extended varying distances from the cell membrane using synthetic alpha helices of varying length. In particular, synthetic oligonucleotides representing repeated alpha helical motifs of lysine and glutamic acid residues flanked by two alanine residues were designed (see Fig. 28 for the primary nucleic acid and amino acid sequences). In previous studies, such amino acid sequences were found to occur with high frequency in alpha helices, suggesting that such repeated motifs would adopt an alpha helical conformation and that placement of such alpha helices between the transmembrane domain and extracellular domains of CD4 would project CD4 away from the cell membrane. By varying the length of the alpha helical segment, a calculation of the projection distance necessary

to resist HIV entry was determined based on known values for alpha helical rise and turn. These results are presented in Table 1.

TABLE 1

	Syncytia Formation	Thy-1 Expression
A. CD4+H+CH2+CH3+CD7tm+stk	-	-
B. CD4(D1,D2)+H+CH2+CH3+CD7tm+stk	-	-
C. CD4+CD7tm+stk	+/- ^(a)	+
D. CD4+CD7tm (long version)	+	+
E. CD4+CD7tm (short version)	+	+
F. CD4+CD5tm	+	+
G. CD4+CH2+CH3+CD5tm	-	-
H. CD4+CH3+CD5tm	-	ND
I. CD4+CD34tm	+	-
J. CD4+synthetic alpha helix (24 angstroms)+CD34tm	ND	-
K. CD4+synthetic alpha helix (48 angstroms)+CD34tm	ND	+/- ^(a)
L. CD4+synthetic alpha helix (72 angstroms)+CD34tm	ND	-

^a Substantial reduction in the number of syncytia or thy-1-expressing cells.

In this Table, "CD4" represents CD4(D1-D4) unless otherwise noted; "H", "CH2", and "CH3" represent the hinge,

CH2, and CH3 regions of the human IgG1 heavy chain,
respectively; "CD7tm and stk" represents the CD7
transmembrane and stalk regions; "CD7tm (long version)" and
"CD7tm (short version)" represent respectively the CD7
5 transmembrane region and the CD7 transmembrane region
deleted for the proline-rich domain (as discussed above);
"CD5tm" represents the CD5 transmembrane region; and
"CD34tm" represents the CD34 transmembrane region. In
entries J-L, the length of the alpha helical region is
10 denoted in angstroms; these values are based on the fact
that there are 3.6 residues per turn of an alpha helix,
corresponding to 5.4 A (or 1.5A per residue). Accordingly,
a 16 residue alpha helix would project the extracellular
domain of CD4 about 24 angstroms. The 48 and 72 angstrom
alpha helices were constructed by sequential
concatemerization of the BstY1 fragment into the fragment's
unique BamH1 site (see Fig. 28), followed by selection of
clones with the proper orientation.

Syncytia formation was scored in co-cultivation
assays with HeLa cells expressing the HIV-1 envelope
glycoprotein from the vaccinia virus vPE-16 construct (see
above).

Thy-1 expression was measured as follows. A live
retrovirus vector was constructed based on the hxb.2 clone
25 of HIV-1. In this vector, the non-essential nef gene was
replaced with the coding sequence of rat thy-1, an
efficiently expressed cell surface molecule that is anchored
to the membrane by a phosphatidyl-inositol linkage. The
virus derived from this molecular clone, designated hxb/thy-
30 1, was infectious as evidenced by its cytopathological
effects and by the production of p24 in culture supernatants
of infected C8166 cells (a human CD4⁺ leukemic T-cell line).
In addition, upon exposure to hxb/thy-1, HeLa cells

transiently transfected with CD4 showed signs of thy-1 expression in as early as 18 hours post-infection, as would be expected of a message regulated in a nef-like manner. Messages encoded by the nef gene normally fall into a class of viral regulatory proteins which are multiply spliced and lack the rev-response element. These messages can accumulate constitutively in the cytoplasm as early viral gene products. The thy-1 messages were expected to be similarly regulated, that is, to occur early in the life cycle of the virus. In short, this system facilitated the assay of HIV entry, with thy-1 expression employed as a surrogate for viral entry. Various CD4-based chimeras were transiently transfected into HeLa cells using standard DEAE-dextran methods. The transfected cells were exposed to hxb/thy-1 virus at 48 hours post-transfection and scored for thy-1 expression at 24-48 hours post-infection. In the results shown in Table 1, thy-1 expression was measured at 24 hours post-infection using a commercially available Thy-1 monoclonal antibody (Accurate).

From the data presented in Table 1, we concluded that the extracellular domains of CD4 should optimally be projected away from the cell membrane by at least 48 angstroms, and preferably by at least 72 angstroms in order to resist HIV-1 infection.

Using a strategy similar to the general strategy described herein, chimeras based on anti-HIV envelope antibodies may be constructed which target HIV-infected cells. Examples of such antibodies are described in Gorny et al., Proc. Natl. Acad. Sci. USA 86:1624 (1989) and Marasco et al., J. Clin. Invest. 90:1467 (1992).

EXAMPLE XI

Additional T Cell Receptor and B Cell Receptor Trigger Proteins

Other intracellular and transmembrane signal transducing domains according to the invention may be derived from the T cell receptor proteins, CD3 delta and T3 gamma, and the B cell receptor proteins, mb1 and B29. The amino acid sequences of these proteins are shown in Fig. 16 (CD3 delta; SEQ ID NO: 24), Fig. 17 (T3 gamma; SEQ ID NO: 25), Fig. 18 (mb1; SEQ ID NO: 26) and Fig. 19 (B29; SEQ ID NO: 27). The portions of the sequences sufficient for cytolytic signal transduction (and therefore preferably included in a chimeric receptor of the invention) are shown in brackets. Chimeric receptors which include these protein domains are constructed and used in the therapeutic methods of the invention generally as described above.

EXAMPLE XII

Experimental Methods

Vaccinia Infection and Radioimmunoprecipitation

Approximately 5×10^6 CV1 cells were infected for one hour in serum free DME medium with recombinant vaccinia at a multiplicity of infection (moi) of at least ten (titer measured on CV1 cells). The cells were placed in fresh medium after infection and labelled metabolically with $200\mu\text{Ci/ml}$ ^{35}S -methionine plus cysteine (Tran ^{35}S -label, ICN; Costo Mesa, CA) in methionine and cysteine free DMEM (Gibco; Grand Island, NY) for six hours. The labelled cells were detached with PBS containing 1mM EDTA, collected by centrifugation, and lysed in 1% NP-40, 0.1% SDS, 0.15 M NaCl, 0.05M Tris pH 8.0, 5mM EDTA, and 1mM PMSF. Nuclei were removed by centrifugation, and CD4 proteins immunoprecipitated with OKT4 antibody and anti-mouse IgG agarose (Cappel, Durham, NC). Samples were electrophoresed

through 8% polyacrylamide/SDS gels under non-reducing (NR) and reducing (R) conditions. Gels containing ^{35}S -labelled samples were impregnated with En^3Hance (New England Nuclear, Boston, MA) prior to autoradiography. Facilitated

5 expression of the transmembrane form of CD16, CD16_{TM} , was measured by comparing its expression in CV1 cells singly infected with CD16_{TM} with expression in cells coinfectd with viruses encoding CD16_{TM} and ζ or γ chimeras. After infection and incubation for six hours or more, cells were
10 detached from plates with PBS, 1mM EDTA and the expression of CD16_{TM} or the chimeras was measured by indirect immunofluorescence and flow cytometry.

Calcium Flux Assay

Jurkat subline E6 (Weiss et al., J. Immunol. 133:123-128 (1984)) cells were infected with recombinant vaccinia viruses for one hour in serum free IMDM at an moi of 10 and incubated for three to nine hours in IMDM, 10% FBS. Cells were collected by centrifugation and resuspended at 3×10^6 cells/ml in complete medium containing 1mM Indo-1 acetomethoxyester (Grynkiewicz et al., J. Biol. Chem. 260:3340-3450 (1985)) (Molecular Probes) and incubated at 37°C for 45 minutes. The Indo-1 loaded cells were pelleted and resuspended at 1×10^6 /ml in serum free IMDM and stored at room temperature in the dark. Cells were analyzed for
25 free calcium ion by simultaneous measurement of the violet and blue fluorescence emission by flow cytometry (Rabinovitch et al., J. Immunol. 137:952-961 (1986)). To initiate calcium flux, either phycoerythrin (PE)-conjugated Leu-3A (anti-CD4) (Secton Dickinson, Lincoln Park, NJ) at 1
30 $\mu\text{g}/\text{ml}$ was added to the cell suspension followed by $10\mu\text{g}/\text{ml}$ of unconjugated goat anti-mouse IgG at time 0 or unconjugated 3G8 (anti-CD16) monoclonal antibody was added to the cell suspension at $1\mu\text{g}/\text{ml}$ followed by $10\mu\text{g}/\text{ml}$ of

PE-conjugated Fab₂' goat anti-mouse IgG at time 0. Histograms of the violet/blue emission ratio were collected from the PE positive (infected) cell population, which typically represented 40-80% of all cells. The T cell antigen receptor response in uninfected cells was triggered by antibody OKT3, without crosslinking. For experiments involving CD16 chimeric receptors, samples showing baseline drift toward lower intracellular calcium (without antibody) were excluded from the analysis. Histogram data were subsequently analyzed by conversion of the binary data to ASCII using Write Hand Man (Cooper City, FL) software, followed by analysis with a collection of FORTRAN programs. The violet/blue emission ratio prior to the addition of the second antibody reagents was used to establish the normalized initial ratio, set equal to unity, and the resting threshold ratio, set so that 10% of the resting population would exceed threshold.

Cytolysis Assay

Human T cell line WH3, a CD8⁺ CD4⁻ HLA B44 restricted cytolytic line was maintained in IMDM, 10% human serum with 100 U/ml of IL-2 and was periodically stimulated either nonspecifically with irradiated (3000 rad) HLA-unmatched peripheral blood lymphocytes and 1µg/ml of phytohemagglutinin, or specifically, with irradiated B44-bearing mononuclear cells. After one day of nonspecific stimulation, the PHA was diluted to 0.5 µg/ml by addition of fresh medium, and after three days the medium was changed. Cells were grown for at least 10 days following stimulation before use in cytotoxicity assays. The cells were infected with recombinant vaccinia at a multiplicity of infection of at least 10 for one hour in serum free medium, followed by incubation in complete medium for three hours. Cells were harvested by centrifugation and resuspended at a density of

1 x 10⁷ cells/ml: 100 μl were added to each well of a U-bottom microtiter plate containing 100 μl/well of complete medium. Cells were diluted in two-fold serial steps. Two wells for each sample did not contain lymphocytes, to allow spontaneous chromium release and total chromium uptake to be measured. The target cells, from HeLa subline S3, were infected in 6.0 or 10.0 cm plates at an approximate moi of 10 for one hour in serum free medium, followed by incubation in complete medium for three hours. They were then detached from the dishes with PBS, 1mM EDTA and counted. An aliquot of 10⁶ target cells (HeLa, Raji, or RJ2.2.5 cells for the CD4 chimeric receptor experiments and 3G8 10-2 cells; Shen et al., Mol. Immunol. 26:959 (1989) for the CD16 chimeric receptor experiments) was centrifuged and resuspended in 50 μl of sterile ⁵¹Cr-sodium chromate (1mCi/ml, Dupont Wilmington, DE) for one hour at 37°C with intermittent mixing, then washed three times with PBS. 100 μl of labelled cells resuspended in medium at 10⁵ cells/ml were added to each well. Raji and RJ2.2.5 target cells were labelled in the same manner as HeLa cells. The microtiter plate was spun at 750 x g for 1 minute and incubated for 4 hours at 37°C. At the end of the incubation period, the cells in each well were resuspended by gentle pipetting, a sample removed to determine the total counts incorporated, and the microtiter plate spun at 750 x g for 1 minute. 100 μl aliquots of supernatant were removed and counted in a gamma ray scintillation counter. The percent killing was corrected for the fraction of infected target cells (usually 50-90%) measured by flow cytometry. For infected effector cells the effector:target ratio was corrected for the percent of cells infected (usually 20-50% for the CD4 chimeric receptor experiments and >70% for the CD16 chimeric receptor experiments).

0003937 062401
104230 1656660

In Vitro Mutagenesis of the ζ Sequence

To create point mutations in amino acid residues 11 and or 15 of the ζ sequence, synthetic oligonucleotide primers extending from the BamHI site upstream of the ζ transmembrane domain, and converting native ζ residue 11 from Cys to Gly (C11G) or residue 15 from Asp to Gly (D15G) or both (C11G/D15G) were prepared and used in PCR reactions to generate mutated fragments which were reinserted into the wild type CD4: ζ constructs.

To create ζ deletions, ζ cDNA sequences were amplified by PCR using synthetic oligonucleotide primers designed to create a stop codon (UAG) after residues 50, 59, or 65. The primers contained the cleavage site for the enzyme NotI indented five or six residues from the 5' end, usually in a sequence of the form CGC GGG CGG CCG CTA (SEQ ID NO: 11), where the last three residues correspond to the stop anticodon. The NotI and stop anticodon sequences were followed by 18 or more residues complementary to the desired 3' end of the fragment. The resulting chimeras were designated CD16: ζ Y51*, CD16: ζ E60* and CD16: ζ D66* respectively. The BamHI site upstream of the transmembrane domain and the NotI site were used to generate fragments that were reintroduced into the wild type CD16: ζ construct. Monomeric ζ chimeras were created by liberating the ζ transmembrane and membrane proximal intracellular sequences by BamHI and SacI digestion of the Asp⁻ and Cys⁻ CD4: ζ construct described above and inserting the fragment into the CD16: ζ E60* and CD16: ζ D66* construct respectively. CD16:7: ζ (48-65) and CD16:7: ζ (48-59) tripartite chimera construction.

To prepare the construct CD16: ζ D66*, the ζ cDNA sequence corresponding to the transmembrane domain and the 17 following residues of the cytoplasmic domain was replaced

0997537-062401
T04230-256560

by corresponding transmembrane and cytoplasmic domain obtained from the CD5 and CD7 cDNA. The CD5 and CD7 fragments were generated by a PCR reaction using forward oligonucleotides including a BamHI restriction cleavage site and corresponding to the region just upstream of the transmembrane domain of CD5 and CD7 respectively and the following reverse oligonucleotides overlapping the CD5 and CD7 sequences respectively and the ζ sequence which contained the SacI restriction cleavage site.

CD5:ζ: CGC GGG CTC GTT ATA GAG CTG GTT CTG GCG CTG CTT CTT CTG (SEQ ID NO: 12)

CD7:ζ: CGC GGG GAG CTC GTT ATA GAG CTG GTT TGC CGC CGA ATT CTT ATC CCG (SEQ ID NO: 13).

The CD5 and CD7 PCR products were digested with BamHI and SacI and ligated to BamHI and SacI digested CD16:ζE60* and replacing the ζ sequence from BamHI to SacI by the CD7 fragment. To make the constructs CD16:CD5 and CD16:CD7, CD5 and CD7 fragments were obtained by PCR using an oligonucleotide containing a NotI restriction cleavage site and encoding a stop codon (UAA) after the residue Gln416 and Ala193 of CD5 and CD7 respectively. The CD5 and CD7 PCR fragment were digested with BamHI and NotI and inserted in the CD16:ζAsp66* construct.

In Vitro Mutagenesis of the N-terminal Residues within the ζ Cytolytic Signal-Transducing Motif

Synthetic oligonucleotide primers extending from the SacI site inside the ζ motif and converting native residue 48 from Asn to Ser (N48S), residue 50 from Leu to Ser (L50S) and residue 51 from Tyr to Phe (Y51F) were synthesized and used in a PCR reaction to generate fragments that were reintroduced into the wild type CD16:7:ζ(48-65) construct.

In Vitro Mutagenesis of C-terminal Residues within the ζ Cytolytic Signal-Transducing Motif

090353.06401
FOI 290 " 255660

5

10

15

25

(SEQ ID NO: 16); and

30

Construction of FcR γ IIA Deletion Mutants

Carboxyl terminal FCRIIA deletion mutants were constructed by PCR in the same fashion as for the full

length constructs, converting the sequences encoding tyrosine at positions 282 and 298 into stop codons (TAA). The N-terminal deletions were generated by amplifying fragments encoding successively less of the intracellular domain by PCR, using oligonucleotides which allowed the resulting fragments to be inserted between MluI and NotI restriction sites into a previously constructed expression plasmid encoding the CD16 extracellular domain fused to the CD7 transmembrane domain, the latter terminating in a MluI site ant the junction between the transmembrane and the intracellular domain.

OTHER EMBODIMENTS

The examples described above demonstrate that aggregation of ζ , η , or γ chimeras suffices to initiate the cytolytic effector cell response in T cells. The known range of expression of ζ , η , and γ , which includes T lymphocytes, natural killer cells, basophilic granulocytes, macrophages and mast cells, suggests that conserved sequence motifs may interact with a sensory apparatus common to cells of hematopoietic origin and that an important component of host defense in the immune system may be mediated by receptor aggregation events.

The potency of the cytolytic response and the absence of a response to target cells bearing MHC class II receptors demonstrates that chimeras based on ζ , η , or γ form the basis for a genetic intervention for AIDS through adoptive immunotherapy. The broad distribution of endogenous ζ and γ and evidence that Fc receptors associated with γ mediate cytotoxicity in different cells types (Fanger et al., Immunol. Today 10:92-99 (1989)) allows a variety of cells to be considered for this purpose. For example, neutrophilic granulocytes, which have a very short lifespan

(\approx 4h) in circulation and are intensely cytolytic, are attractive target cells for expression of the chimeras. Infection of neutrophils with HIV is not likely to result in virus release, and the abundance of these cells (the most prevalent of the leukocytes) should facilitate host defense. Another attractive possibility for host cells are mature T cells, a population presently accessible to retroviral engineering (Rosenberg, S.A. Sci. Am. 262:62-69 (1990)). With the aid of recombinant IL-2, T cell populations can be expanded in culture with relative ease, and the expanded populations typically have a limited lifespan when reinfused (Rosenberg et al., N. Engl. J. Med. 323:570-578 (1990)).

Under the appropriate conditions, HIV recognition by cells expressing CD4 chimeras should also provide mitogenic stimuli, allowing the possibility that the armed cell population could respond dynamically to the viral burden. Although we have focused here on the behavior of the fusion proteins in cytolytic T lymphocytes, expression of the chimeras in helper lymphocytes might provide an HIV-mobilized source of cytokines which could counteract the collapse of the helper cell subset in AIDS. Recent description of several schemes for engineering resistance to infection at steps other than virus penetration (Friedman et al., Nature 335:452-454 (1988); Green et al., Cell 58:215-223 (1989); Malim et al., Cell 58:205-214 (1989); Trono et al., Cell 59:113-120 (1989); Buonocore et al., Nature 345:625-628 (1990)) suggests that cells bearing CD4 chimeras could be designed to thwart virus production by expression of appropriate agents having an intracellular site of action.

The ability to transmit signals to T lymphocytes through autonomous chimeras also provides the ability for the regulation of retrovirally engineered lymphocytes in

vivo. Crosslinking stimuli, mediated for example by specific IgM antibodies engineered to remove complement-binding domains, may allow such lymphocytes to increase in number in situ, while treatment with similar specific IgG antibodies (for example recognizing an amino acid variation engineered into the chimeric chain) could selectively deplete the engineered population. Additionally, anti-CD4 IgM antibodies do not require additional crosslinking to mobilize calcium in Jurkat cells expressing CD4:ζ chimeras. The ability to regulate cell populations without recourse to repeated extracorporeal amplification may substantially extend the range and efficacy of current uses proposed for genetically engineered T cells.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover variations, uses, or adaptations of the invention and including such departures from the present disclosure as come within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

104280 255660